REMARKS

Claims 34 and 88 to 133 were pending in the subject application. Applicants have herein canceled claims 34 and 88 to 133 without prejudice or disclaimer of applicants' rights to pursue the subject matter of these claims in this or another application, and have added claims 134 to 154.

Support for New Claims

The subject application is a continuation of U.S. Serial No. 10/346,853, filed January 17, 2003, which is a continuation of U.S. Serial No. 09/100,812, filed June 19, 1998, now U.S. Patent No. 6,573,099 B2, issued June 3, 2003, which claims priority of Australian Provisional Patent Application No. PP2492, filed March 20, 1998 (the "Priority Application"). The new claims are fully supported in each disclosure.

Independent claim 134

a) "producing an RNA molecule" and "transcribed"

Support for this language may be found, *inter alia*, in each specification at page 7, lines 5 to 6; and page 18, lines 27 to 28.

b) "capable of delaying, repressing or otherwise reducing the expression of a target gene"

Support for this language may be found, *inter alia*, in each specification at page 1A, lines 7 to 9.

c) "mammalian cell"

Support for this language may be found, *inter alia*, in each specification at page 22, lines 19 to 21.

d) "introducing into a cell"

Support for this language may be found, *inter alia*, in each specification at page 23, lines 9 to 15.

e) "double-stranded"

Examples of a "double-stranded" synthetic genes are replete in each disclosure. Specifically, a number of genetic constructs are described in each disclosure. *See* page 28, line 14, to page 39, line 22. The genetic constructs that each specification describes are ultimately derived from a double-stranded plasmid, such as pCR2.1. *See, e.g.*, page 27, lines 1 to 8. Applicants attach hereto as **Exhibit A** a map of plasmid pCR2.1, which is a commercially available starting plasmid for a number of the "Examples". Additionally, each specification describes blunt-ended fragments. *See, e.g.*, page 37, lines 6 to 7.

f) "synthetic gene"

Support for this language may be found, *inter alia*, in each specification at page 7, lines 1 to 3.

g) "operable in the cell"

Support for this language may be found, *inter alia*, in each specification at page 3, line 16.

h) "transcription termination sequence"

Support for this language may be found, *inter alia*, in each specification at page 22, lines 12 to 14.

i) "active in the cell"

Support for this language may be found, *inter alia*, in each specification at page 22, lines 19 to 20.

j) "operable connected thereto"

Support for this language may be found, *inter alia*, in each specification at page 11, lines 28 to 29.

k) "first and second structural gene sequences"

Support for this language may be found, *inter alia*, in each specification at page 3, lines 9 to 16; page 7, lines 5 to 7; page 16, lines 20 to 26; and page 18, lines 16 to 20.

l) "20-30 consecutive nucleotides in length"

Support for this language may be found, *inter alia*, in each specification at page 10, lines 15 to 17.

m) "identical"

Support for this language may be found, *inter alia*, in each specification at page 3, lines 12 to 14; page 8, lines 14 to 22; and page 18, lines 17 to 18.

n) "inverted orientation"

Support for this language may be found, *inter alia*, in each specification at page 18, lines 16 to 20.

o) "repeating sequence"

Support for this language may be found, *inter alia*, in each specification at page 18, lines 16 to 20.

p) "stuffer fragment"

Support for this language may be found, *inter alia*, in each specification at page 19, lines 14 to 22.

Support for dependent claims

Support for new claim 135 may be found, *inter alia*, in each specification at page 16, line 26, to page 17, line 2.

Support for new claim 136 may be found, *inter alia*, in each specification at page 10, lines 10 to 12.

Support for new claim 137 may be found, *inter alia*, in each specification at page 10, lines 15 to 17.

Support for new claim 138 may be found, *inter alia*, in each specification at page 7, lines 25 to 27.

Support for new claim 139 may be found, *inter alia*, in each specification at page 7, lines 25 to 28.

Support for new claim 140 may be found, *inter alia*, in each

specification at page 7, lines 25 to 26.

Support for new claim 141 may be found, *inter alia*, in each specification at page 7, lines 25 to 29.

Support for new claim 142 may be found, *inter alia*, in each specification at page 7, lines 18 to 20.

Support for new claim 143 may be found, *inter alia*, in each specification at page 7, lines 18 to 19.

Support for new claim 144 may be found, *inter alia*, in each specification at page 12, lines 1 to 3.

Support for new claim 145 may be found, *inter alia*, in each specification at page 12, lines 1 to 2.

Support for new claim 146 may be found, *inter alia*, in each specification at page 18, lines 16 to 20.

Support for new claims 147 and 148 may be found, *inter alia*, in each specification at page 19, lines 24 to 25.

Support for new claim 149 may be found, *inter alia*, in each specification at page 19, lines 24 to 26.

Support for new claims 150 and 151 may be found, *inter alia*, in each specification at page 23, lines 9 to 11.

Support for new claim 152 may be found, *inter alia*, in each specification at page 24, lines 27 to 29.

Support for new claim 153 may be found, *inter alia*, in each specification at page 22, lines 19 to 21.

Support for new claim 154 may be found, *inter alia*, in each specification at page 23, lines 21 to 24.

Applicants have herein canceled claims 34 and 88 to 133 without prejudice or disclaimer of applicants' rights to pursue the subject matter of these claims in this or another application. Accordingly, after entry of this Amendment, claims 134 to 154 will be pending and under examination.

The November 2, 2007 Final Office Action

The November 2, 2007 Final Office Action rejected claims 24 and 88 to 133 as allegedly unpatentable over U.S. Patent No. 6,506,559 B1 ("Fire et al. Patent") in view of PCT International Application No. WO 94/01550 A1 ("Agrawal et al."). Without acknowledging the accuracy of the Examiner's position, applicants have canceled claims 34 and 88 to 133 without prejudice to applicants' right to pursue the subject matter of these claims in this or a related application, and have added claims 134 to 154.

Applicants maintain that the rejection of claims 34 and 88 to 133 in the November 2, 2007 Final Office Action cannot be applied to the pending claims at least for the reasons given below.

1. Fire et al. Patent is not prior art to the claimed invention

As an initial matter, applicants maintain that Fire et al. Patent is not prior art to the subject application. To summarize, the amended claims are entitled to the priority of the March 20, 1998

filing date of Australian Provisional Patent Application No. PP2492. Fire et al. Patent issued from an application submitted to the United States Patent and Trademark Office on December 23, 1998, *i.e.* after the priority date of the subject application.

Fire et al. Patent claims the benefit of U.S. Provisional Application No. 60/068,562, filed December 23, 1997 ("Fire et al. Provisional"). However, Fire et al. Provisional discloses less than Fire et al. Patent. Applicants attach hereto as **Exhibit B** a copy of Fire et al. Patent marked-up to show differences from Fire et al. Provisional. Applicants respectfully maintain that any rejection which relies on disclosures not in Fire et al. Provisional are clearly improper.

2. The combination of Fire et al. Provisional and Agrawal et al. does not teach or suggest a double-stranded synthetic gene comprising a "a repeating sequence of 20-30 consecutive nucleotides in length" as claimed

The combination of Fire et al. Provisional and Agrawal et al. cannot render obvious the pending claims because the combination does not teach or suggest double-stranded synthetic genes having a repeated sequence of only "20-30 consecutive nucleotides" in length identical to the target gene as claimed. Applicants respectfully maintain that the rejection of the previously presented claims cannot be applied to the amended claims for this reason alone.

3. The aforementioned feature of the pending claims imparts an unpredictable advantage to the claimed invention

Double-stranded synthetic genes of the claimed process comprise "20-30 consecutive nucleotides" "identical in sequence to a region of a target gene". This is an optimal range *in mammalian*

cells. Applicants attach hereto a copy of Paul, C.P., et al., (2002) "Effective expression of small interfering RNA in human cells," Nat. Biotechol. 20(5):505-508 as **Exhibit C** which was published after the filing date of the Priority Application. Paul et al. indicates that:

> "The use of this 'RNA interference' (RNAi) in mammalian studies had lagged well behind its utility in lower animals because uninterrupted RNA duplexes longer than 30 base pairs trigger generalized cellular responses through activation of dsRNA-dependent protein kinases. Recently it was demonstrated that RNAi can be made to work in cultured human cells by introducing shorter, synthetic duplex RNAs (~20 base pairs) through liposome transfection." (Abstract, lines 6 to 11; internal references omitted; emphasis added)

In regard to the transcripts of the double-stranded synthetic genes of the claimed invention, applicants maintain that Paul et al. described i) 20 consecutive nucleotides (single underline) as the lower limit of the optimum range of identity to a target gene in mammalian cells; and ii) 30 consecutive nucleotides as the upper limit of the optimum range of identity to a target gene in mammalian cells. Applicants further maintain this optimal range in mammalian cells as recited in the pending claims could not have been predicted from the prior art generally or in the prior art.

Applicants' priority document as filed teaches that the recited range is preferred. *See, e.g.*, page 10, lines 15 to 17, of Australian Provisional Application No. PP 2492. *See, also*, M.P.E.P. § 716.02(f) (8th Ed., 6th Rev., Sept. 2007).

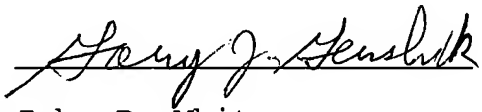
Accordingly, applicants maintain that the rejection of the previously presented claims under 35 U.S.C. § 103(a) as allegedly

obvious over Fire et al. Patent taken with Agrawal et al. cannot be applied to the pending claims.

If a telephone interview would be of assistance in advancing prosecution of the subject application, applicants' undersigned attorney invites the Examiner to telephone him at the number provided below.

No fee, other than the concurrently submitted fee of $810.00 for submitting a Request For Continued Examination (RCE) and the enclosed fee of $460.00 for a two-month extension of time, is deemed necessary in connection with the submission of this Amendment. However, if any fee is required authorization is hereby given to charge the amount of any such fee to Deposit Account No. 03-3125.

Respectfully submitted,



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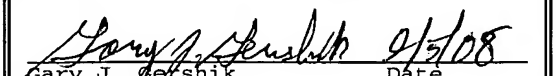
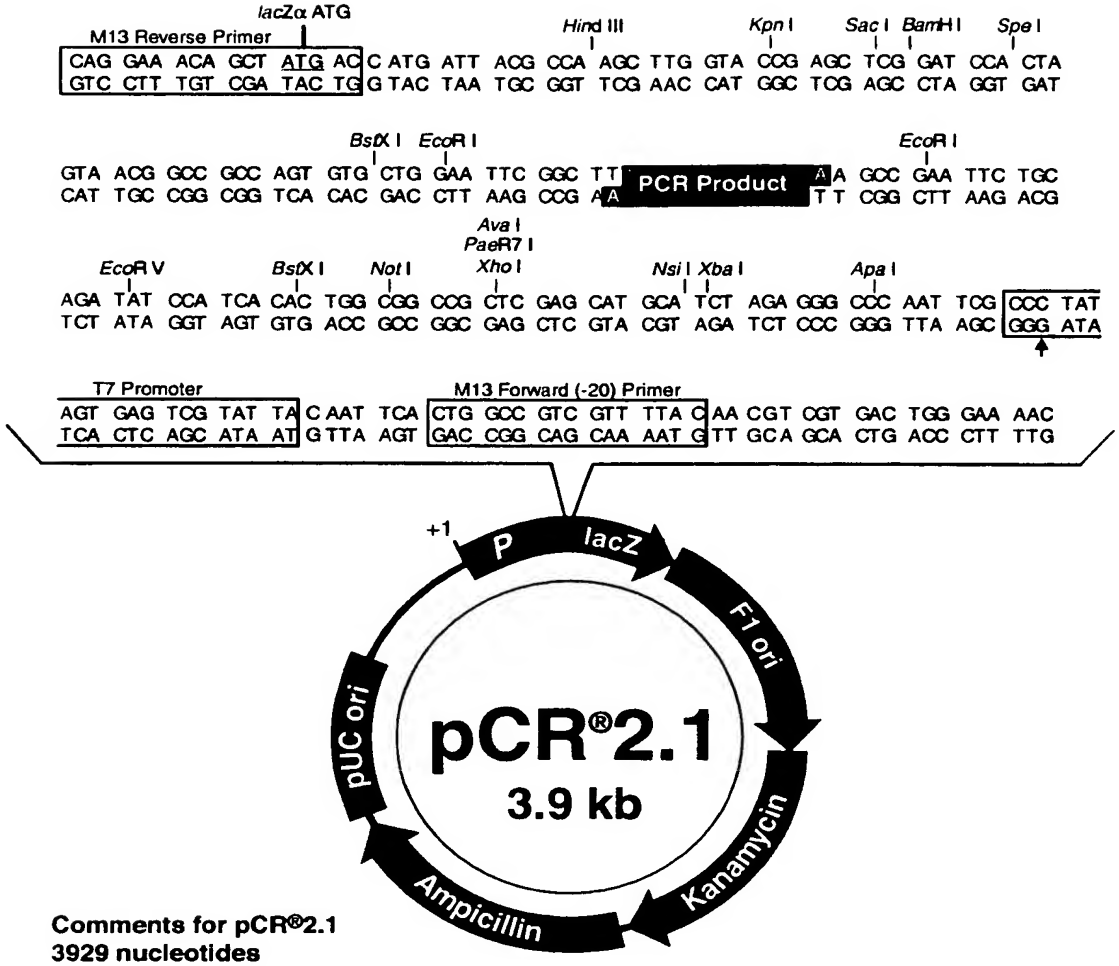


Gary J. Gershik Date
Reg. No. 39,992

EXHIBIT A



Comments for pCR®2.1
3929 nucleotides

LacZα gene: bases 1-545
M13 Reverse priming site: bases 205-221
T7 promoter: bases 362-381
M13 (-20) Forward priming site: bases 389-404
f1 origin: bases 546-983
Kanamycin resistance ORF: bases 1317-2111
Ampicillin resistance ORF: bases 2129-2989
pUC origin: bases 3134-3807

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Applicants: Michael Wayne Graham et al.
U.S. Serial No.: 10/821,726
Filed: April 8, 2004

Exhibit A

EXHIBIT B

US006506559B1

(12) **United States Patent**
Fire et al.

(10) **Patent No.: US 6,506,559 B1**
(45) **Date of Patent: *Jan. 14, 2003**

(54) **GENETIC INHIBITION BY DOUBLE-STRANDED RNA**

(75) Inventors: **Andrew Fire**, Baltimore, MD (US); **Stephen Kostas**, Chicago, IL (US); **Mary Montgomery**, St. Paul, MN (US); **Lisa Timmons**, Lawrence, KS (US); **SiQun Xu**, Ballwin, MO (US); **Hiroaki Tabara**, Shizuoka (JP); **Samuel E. Driver**, Providence, RI (US); **Craig C. Mello**, Shrewsbury, MA (US)

(73) Assignee: **Carnegie Institute of Washington**, Washington, DC (US)

(*) Notice: This patent issued on a continued prosecution application filed under 37 CFR 1.53(d), and is subject to the twenty year patent term provisions of 35 U.S.C. 154(a)(2).

Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/215,257**

(22) Filed: **Dec. 18, 1998**

Related U.S. Application Data

(60) Provisional application No. 60/068,562, filed on Dec. 23, 1997.

(51) **Int. Cl.**[7] **C12Q 1/68**; C12N 15/85
(52) **U.S. Cl.** **435/6**; 435/91.1; 435/325
(58) **Field of Search** 514/44; 435/6, 435/91.1, 325

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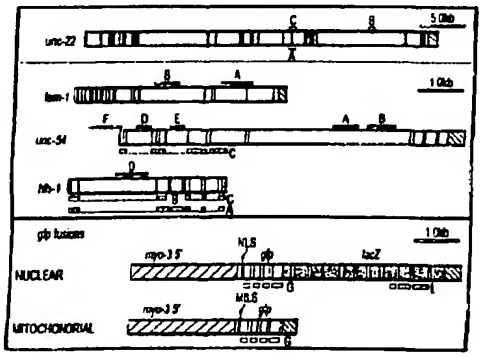
(List continued on next page.)

Primary Examiner—Andrew Wang
Assistant Examiner—Karen A Lacourciere
(74) *Attorney, Agent, or Firm*—Morgan, Lewis & Bockius LLP

(57) **ABSTRACT**

A process is provided of introducing an RNA into a living cell to inhibit gene expression of a target gene in that cell. The process may be practiced ex vivo or in vivo. The RNA has a region with double-stranded structure. Inhibition is sequence-specific in that the nucleotide sequences of the duplex region of the RNA and of a portion of the target gene are identical. The present invention is distinguished from prior art interference in gene expression by antisense or triple-strand methods.

22 Claims, 5 Drawing Sheets



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U.S. Serial No.: 10/821,726
Filed: April 8, 2004

Exhibit B

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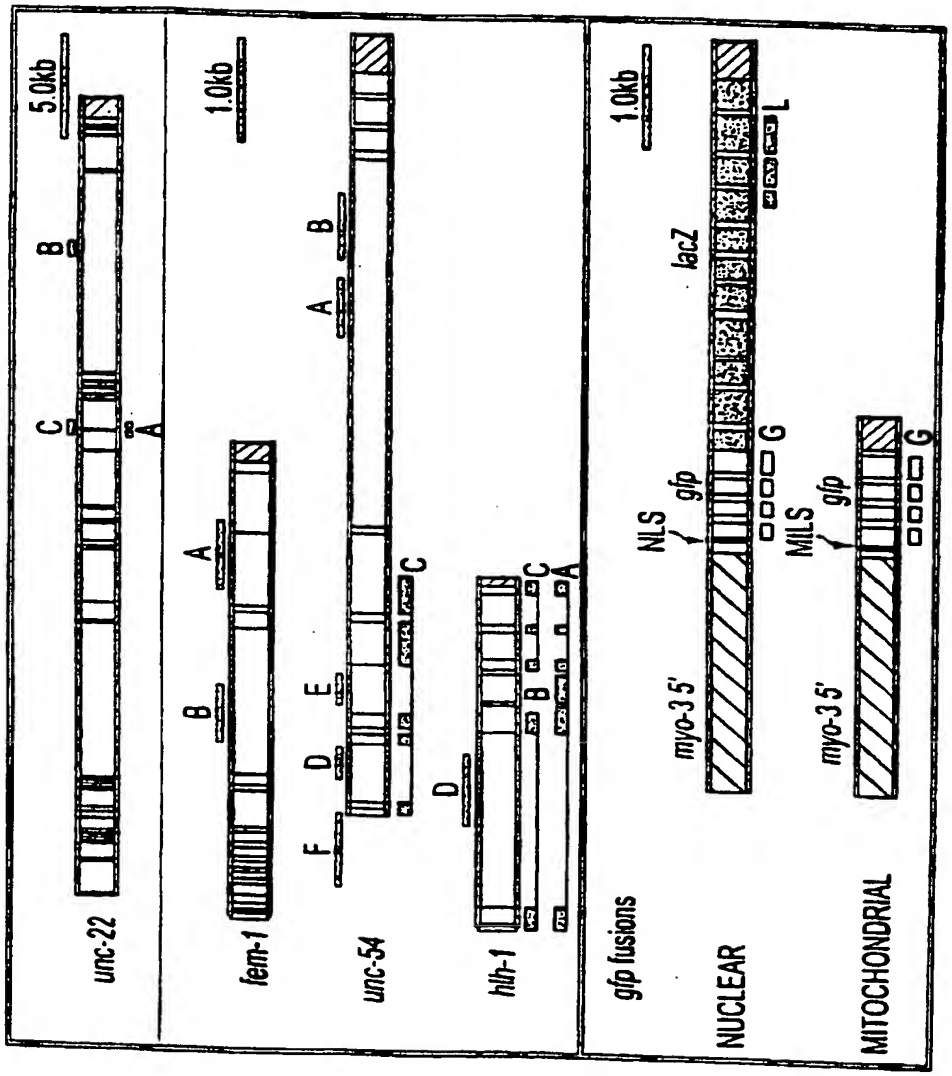
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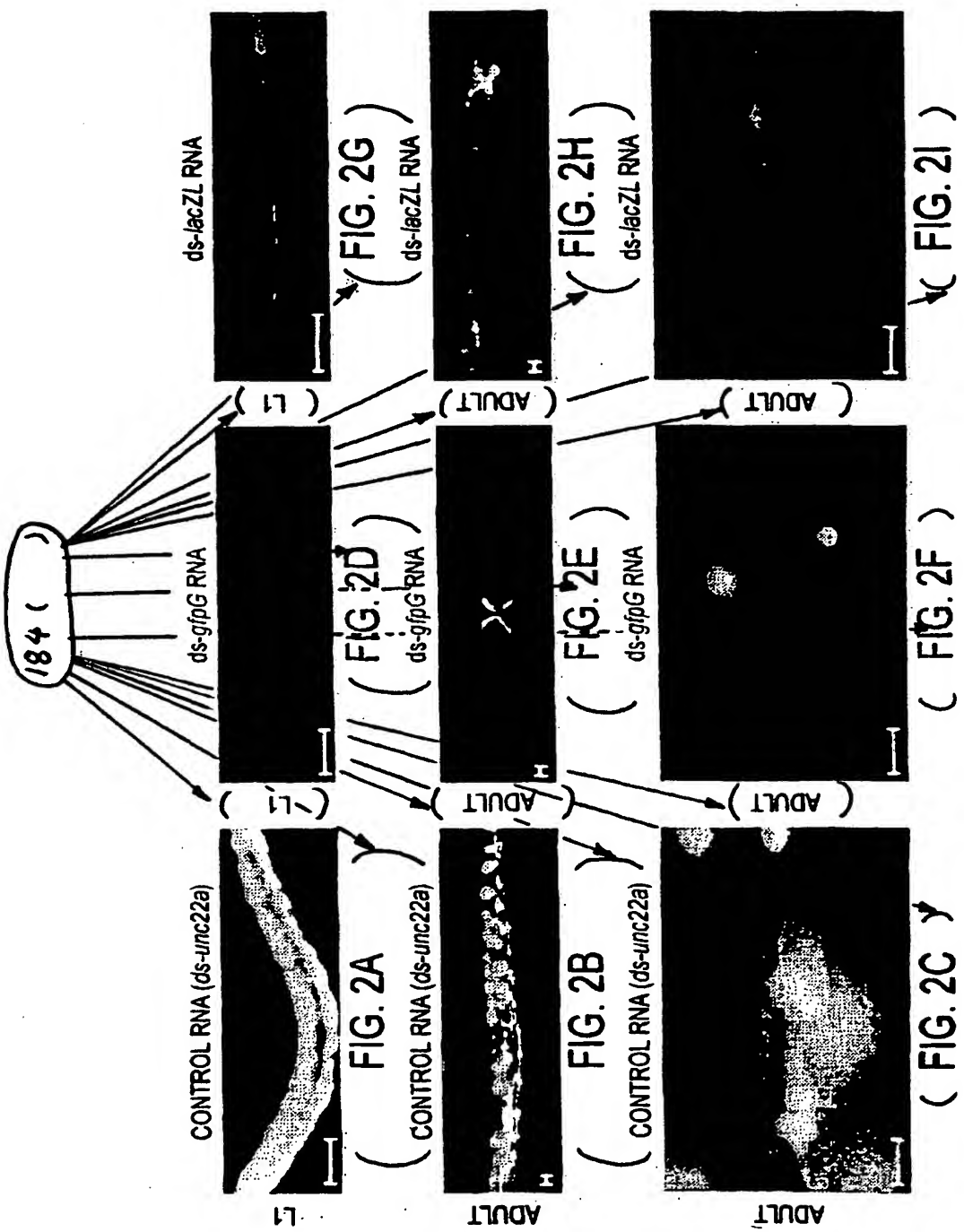
B
C
A
unc-22
1.0kb
B
A
fem-1
F
D
E
A
B
C
unc-54
D
hlh-1
B
C
A
1.0kb
gfp fusions
NLS
gfp
myo-3 5'
lacZ
G
L
NUCLEAR
MLS
gfp
myo-3 5'
G
MITOCHONDRIAL

FIG. 1


184
CONTROL RNA (ds-unc22a)
ds-gfpG RNA
ds-lacZL RNA
L1
ADULT
FIG. 2A
FIG. 2B
FIG. 2C
FIG. 2D
FIG. 2E
FIG. 2F
FIG. 2G
FIG. 2H
FIG. 2I

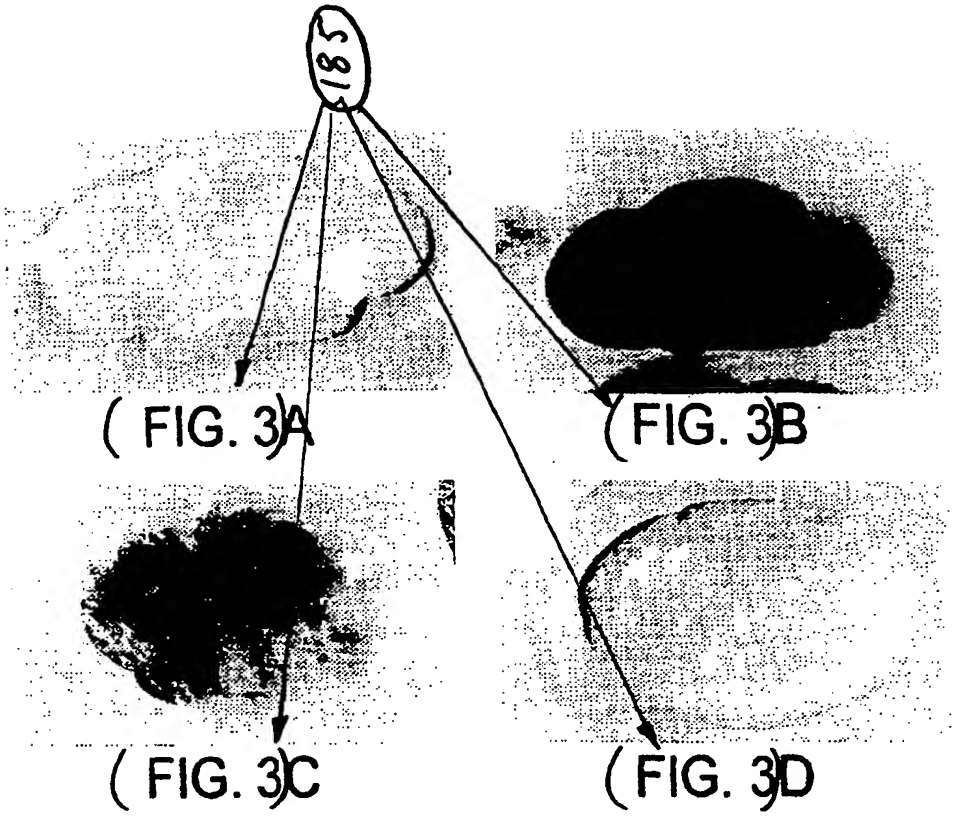
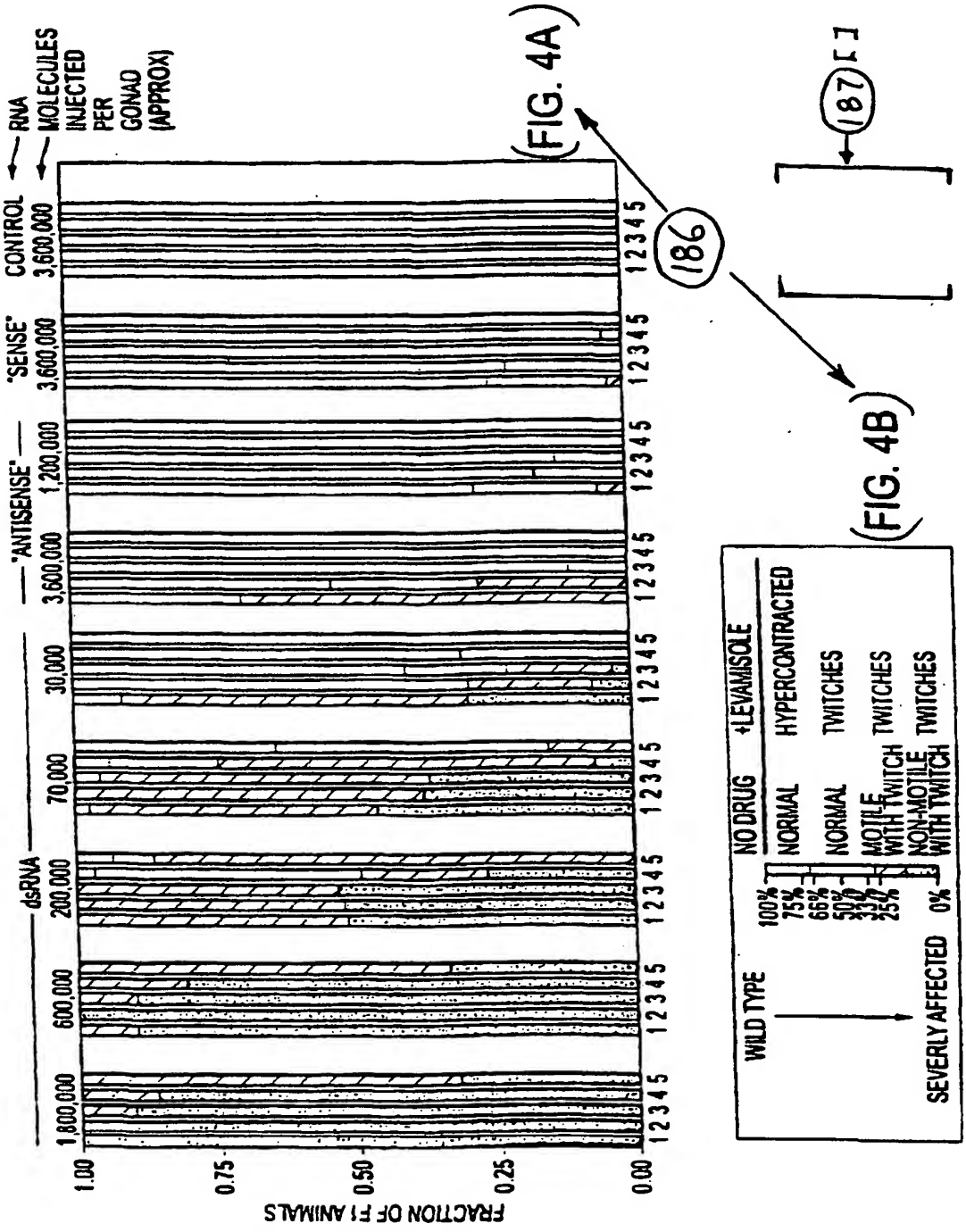


FIG. 3A FIG. 3B

FIG. 3C FIG. 3D


RNA MOLECULES INJECTED PER GONAD (APPROX)
CONTROL
"SENSE"
"ANTISENSE"
dsRNA
3,600,000
1,200,000
30,000
70,000
200,000
600,000
1,800,000
12345
1.00
0.75
0.50
0.25
0.00
FRACTION OF F1 ANIMALS
WILD TYPE
SEVERLY AFFECTED
100%
75%
66%
50%
33%
25%
0%
NO DRUG
+LEVAMISOLE
NORMAL
HYPERCONTRACTED
NORMAL
TWITCHES
MOTILE WITH TWITCH
TWITCHES
NON-MOTILE WITH TWITCH
TWITCHES
186
187
(FIG. 4A)
(FIG. 4B)

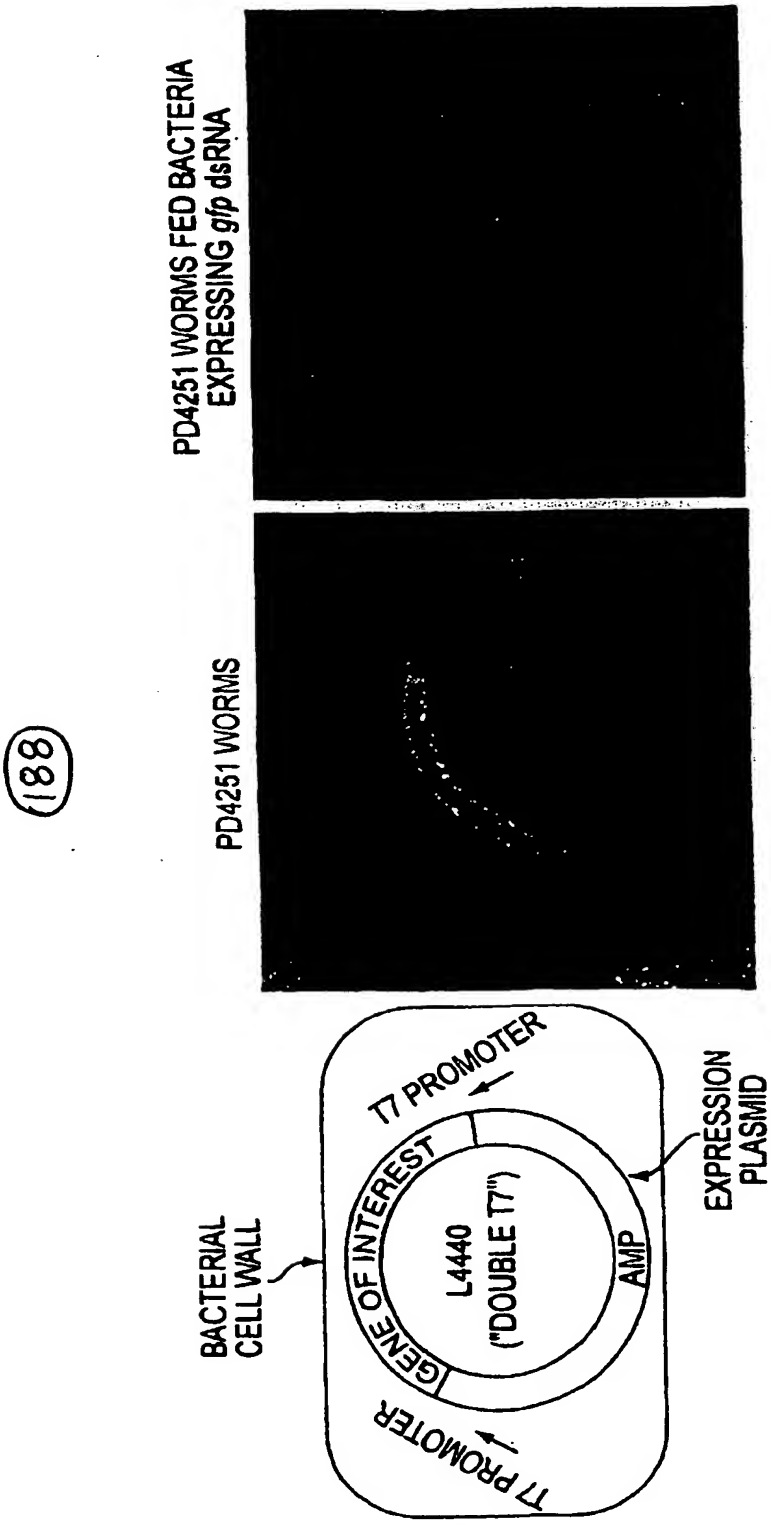


FIG. 5C

FIG. 5B

FIG. 5A

GENETIC INHIBITION BY DOUBLE-STRANDED RNA

RELATED APPLICATION

This application claims the benefit of U.S. Provisional Appln. No. 60/068,562, filed Dec. 23, 1997. +gi

GOVERNMENT RIGHTS

This invention was made with U.S. government support under grant numbers GM-37706, GM-17164, HD-33769 and GM-07231 awarded by the National Institutes of Health. The U.S. government has certain rights in the invention.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to gene-specific inhibition of gene expression by double-stranded ribonucleic acid (dsRNA).

2. Description of the Related Art

Targeted inhibition of gene expression has been a long-felt need in biotechnology and genetic engineering. Although a major investment of effort has been made to achieve this goal, a more comprehensive solution to this problem was still needed.

Classical genetic techniques have been used to isolate mutant organisms with reduced expression of selected genes. Although valuable, such techniques require laborious mutagenesis and screening programs, are limited to organisms in which genetic manipulation is well established (e.g., the existence of selectable markers, the ability to control genetic segregation and sexual reproduction), and are limited to applications in which a large number of cells or organisms can be sacrificed to isolate the desired mutation. Even under these circumstances, classical genetic techniques can fail to produce mutations in specific target genes of interest, particularly when complex genetic pathways are involved. Many applications of molecular genetics require the ability to go beyond classical genetic screening techniques and efficiently produce a directed change in gene expression in a specified group of cells or organisms. Some such applications are knowledge-based projects in which it is of importance to understand what effects the loss of a specific gene product (or products) will have on the behavior of the cell or organism. Other applications are engineering based, for example, cases in which is important to produce a population of cells or organisms in which a specific gene product (or products) has been reduced or removed. A further class of applications is therapeutically based in which it would be valuable for a functioning organism (e.g., a human) to reduce or remove the amount of a specified gene product (or products). Another class of applications provides a disease model in which a physiological function in a living organism is genetically manipulated to reduce or remove a specific gene product (or products) without making a permanent change in the organism's genome.

In the last few years, advances in nucleic acid chemistry and gene transfer have inspired new approaches to engineer specific interference with gene expression. These approaches are described below.

Use of Antisense Nucleic Acids to Engineer Interference

Antisense technology has been the most commonly described approach in protocols to achieve gene-specific interference. For antisense strategies, stochiometric amounts of single-stranded nucleic acid complementary to the messenger RNA for the gene of interest are introduced into the cell. Some difficulties with antisense-based approaches relate to delivery, stability, and dose requirements. In general, cells do not have an uptake mechanism for single-stranded nucleic acids, hence uptake of unmodified single-stranded material is extremely inefficient. While waiting for uptake into cells, the single-stranded material is subject to degradation. Because antisense interference requires that the interfering material accumulate at a relatively high concentration (at or above the concentration of endogenous mRNA), the amount required to be delivered is a major constraint on efficacy. As a consequence, much of the effort in developing antisense technology has been focused on the production of modified nucleic acids that are both stable to nuclease digestion and able to diffuse readily into cells. The use of antisense interference for gene therapy or other whole-organism applications has been limited by the large amounts of oligonucleotide that need to be synthesized from non-natural analogs, the cost of such synthesis, and the difficulty even with high doses of maintaining a sufficiently concentrated and uniform pool of interfering material in each cell.

Triple-Helix Approaches to Engineer Interference

A second, proposed method for engineered interference is based on a triple helical nucleic acid structure. This approach relies on the rare ability of certain nucleic acid populations to adopt a triple-stranded structure. Under physiological conditions, nucleic acids are virtually all single- or double-stranded, and rarely if ever form triple-stranded structures. It has been known for some time, however, that certain simple purine- or pyrimidine-rich sequences could form a triple-stranded molecule in vitro under extreme conditions of pH (i.e., in a test tube). Such structures are generally very transient under physiological conditions, so that simple delivery of unmodified nucleic acids designed to produce triple-strand structures does not yield interference. As with antisense, development of triple-strand technology for use in vivo has focused on the development of modified nucleic acids that would be more stable and more readily absorbed by cells in vivo. An additional goal in developing this technology has been to produce modified nucleic acids for which the formation of triple-stranded material proceeds effectively at physiological pH.

Co-Suppression Phenomena and Their Use in Genetic Engineering

A third approach to gene-specific interference is a set of operational procedures grouped under the name "co-suppression". This approach was first described in plants and refers to the ability of transgenes to cause silencing of an unlinked but homologous gene. More recently, phenomena similar to co-suppression have been reported in two animals: *C. elegans* and Drosophila. Co-suppression was first observed by accident, with reports coming from groups using transgenes in attempts to achieve over-expression of a potentially useful locus. In some cases the over-expression was successful while, in many others, the result was opposite from that expected. In those cases, the transgenic plants actually showed less expression of the endogenous gene. Several mechanisms have so far been proposed for transgene-mediated co-suppression in plants; all of these mechanistic proposals remain hypothetical, and no definitive mechanistic description of the process has been presented. The models that have been proposed to explain co-suppression can be placed in two different categories. In one set of proposals, a direct physical interaction at the DNA- or chromatin-level between two different chromosomal sites has been hypothesized to occur; an as-yet-unidentified mechanism would then lead to de novo methylation and subsequent suppression of gene expression.

Alternatively, some have postulated an RNA intermediate, synthesized at the transgene locus, which might then act to produce interference with the endogenous gene. The characteristics of the interfering RNA, as well as the nature of the interference process, have not been determined. Recently, a set of experiments with RNA viruses have provided some support for the possibility of RNA intermediates in the interference process. In these experiments, a replicating RNA virus is modified to include a segment from a gene of interest. This modified virus is then tested for its ability to interfere with expression of the endogenous gene. Initial results with this technique have been encouraging, however the properties of the viral RNA that are responsible for interference effects have not been determined and, in any case, would be limited to plants which are hosts of the plant virus.

Distinction Between the Present Invention and Antisense Approaches

The present invention differs from antisense-mediated interference in both approach and effectiveness. Antisense-mediated genetic interference methods have a major challenge: delivery to the cell interior of specific single-stranded nucleic acid molecules at a concentration that is equal to or greater than the concentration of endogenous mRNA. Double-stranded RNA-mediated inhibition has advantages both in the stability of the material to be delivered and the concentration required for effective inhibition. Below, we disclose that in the model organism *C. elegans*, the present invention is at least 100-fold more effective than an equivalent antisense approach (i.e., dsRNA is at least 100-fold more effective than the injection of purified antisense RNA in reducing gene expression). These comparisons also demonstrate that inhibition by double-stranded RNA must occur by a mechanism distinct from antisense interference.

Distinction Between the Present Invention and Triple-Helix Approaches

The limited data on triple strand formation argues against the involvement of a stable triple-strand intermediate in the present invention. Triple-strand structures occur rarely, if at all, under physiological conditions and are limited to very unusual base sequence with long runs of purines and pyrimidines. By contrast, dsRNA-mediated inhibition occurs efficiently under physiological conditions, and occurs with a wide variety of inhibitory and target nucleotide sequences. The present invention has been used to inhibit expression of 18 different genes, providing phenocopies of null mutations in these genes of known function. The extreme environmental and sequence constraints on triple-helix formation make it unlikely that dsRNA-mediated inhibition in *C. elegans* is mediated by a triple-strand structure.

Distinction Between Present Invention and Co-Suppression Approaches

The transgene-mediated genetic interference phenomenon called co-suppression may include a wide variety of different processes. From the viewpoint of application to other types of organisms, the co-suppression phenomenon in plants is difficult to extend. A confounding aspect in creating a general technique based on co-suppression is that some transgenes in plants lead to suppression of the endogenous locus and some do not. Results in *C. elegans* and Drosophila indicate that certain transgenes can cause interference (i.e., a quantitative decrease in the activity of the corresponding endogenous locus) but that most transgenes do not produce such an effect. The lack of a predictable effect in plants, nematodes, and insects greatly limits the usefulness of simply adding transgenes to the genome to interfere with gene expression. Viral-mediated co-suppression in plants appears to be quite effective, but has a number of drawbacks. First, it is not clear what aspects of the viral structure are critical for the observed interference. Extension to another system would require discovery of a virus in that system which would have these properties, and such a library of useful viral agents are not available for many organisms. Second, the use of a replicating virus within an organism to effect genetic changes (e.g., long- or short-term gene therapy) requires considerably more monitoring and oversight for deleterious effects than the use of a defined nucleic acid as in the present invention.

The present invention avoids the disadvantages of the previously-described methods for genetic interference. Several advantages of the present invention are discussed below, but numerous others will be apparent to one of ordinary skill in the biotechnology and genetic engineering arts.

SUMMARY OF THE INVENTION

A process is provided for inhibiting expression of a target gene in a cell. The process comprises introduction of RNA with partial or fully double-stranded character into the cell or into the extracellular environment. Inhibition is specific in that a nucleotide sequence from a portion of the target gene is chosen to produce inhibitory RNA. We disclose that this process is (1) effective in producing inhibition of gene expression, (2) specific to the targeted gene, and (3) general in allowing inhibition of many different types of target gene.

The target gene may be a gene derived from the cell, an endogenous gene, a transgene, or a gene of a pathogen which is present in the cell after infection thereof. Depending on the particular target gene and the dose of double stranded RNA material delivered, the procedure may provide partial or complete loss of function for the target gene. A reduction or loss of gene expression in at least 99% of targeted cells has been shown. Lower doses of injected material and longer times after administration of dsRNA may result in inhibition in a smaller fraction of cells. Quantitation of gene expression in a cell may show similar amounts of inhibition at the level of accumulation of target mRNA or translation of target protein.

The RNA may comprise one or more strands of polymerized ribonucleotide; it may include modifications to either the phosphate-sugar backbone or the nucleoside. The double-stranded structure may be formed by a single self-complementary RNA strand or two complementary RNA strands. RNA duplex formation may be initiated either inside or outside the cell. The RNA may be introduced in an amount which allows delivery of at least one copy per cell. Higher doses of double-stranded material may yield more effective inhibition. Inhibition is sequence-specific in that nucleotide sequences corresponding to the duplex region of the RNA are targeted for genetic inhibition. RNA containing a nucleotide sequences identical to a portion of the target gene is preferred for inhibition. RNA sequences with insertions, deletions, and single point mutations relative to the target sequence have also been found to be effective for inhibition. Thus, sequence identity may optimized by alignment algorithms known in the art and calculating the percent difference between the nucleotide sequences. Alternatively, the duplex region of the RNA may be defined functionally as a nucleotide sequence that is capable of hybridizing with a portion of the target gene transcript.

The cell with the target gene may be derived from or contained in any organism (e.g., plant, animal, protozoan, virus, bacterium, or fungus). RNA may be synthesized either in vivo or in vitro. Endogenous RNA polymerase of the cell may mediate transcription in vivo, or cloned RNA polymerase can be used for transcription in vivo or in vitro. For

transcription from a transgene in vivo or an expression construct a regulatory region may be used to transcribe the RNA strand (or strands).

The RNA may be directly introduced into the cell (i.e., intracellularly); or introduced extracellularly into a cavity, interstitial space, into the circulation of an organism, introduced orally, or may be introduced by bathing an organism in a solution containing RNA. Methods for oral introduction include direct mixing of RNA with food of the organism, as well as engineered approaches in which a species that is used as food is engineered to express an RNA, then fed to the organism to be affected. Physical methods of introducing nucleic acids include injection directly into the cell or extracellular injection into the organism of an RNA solution.

The advantages of the present invention include: the ease of introducing double-stranded RNA into cells, the low concentration of RNA which can be used, the stability of double-stranded RNA, and the effectiveness of the inhibition. The ability to use a low concentration of a naturally-occurring nucleic acid avoids several disadvantages of antisense interference. This invention is not limited to in vitro use or to specific sequence compositions, as are techniques based on triple-strand formation. And unlike antisense interference, triple-strand interference, and co-suppression, this invention does not suffer from being limited to a particular set of target genes, a particular portion of the target gene's nucleotide sequence, or a particular transgene or viral delivery method. These concerns have been a serious obstacle to designing general strategies according to the prior art for inhibiting gene expression of a target gene of interest.

Furthermore, genetic manipulation becomes possible in organisms that are not classical genetic models. Breeding and screening programs may be accelerated by the ability to rapidly assay the consequences of a specific, targeted gene disruption. Gene disruptions may be used to discover the function of the target gene, to produce disease models in which the target gene are involved in causing or preventing a pathological condition, and to produce organisms with improved economic properties.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the genes used to study RNA-mediated genetic inhibition in *C. elegans*. Intron-exon structure for genes used to test RNA-mediated inhibition are shown (exons: filled boxes; introns: open boxes; 5' and 3' untranslated regions: shaded; unc-22[9], unc-54[12], fem-1[14], and hlh-1[15]).

FIGS. 2A-I show analysis of inhibitory RNA effects in individual cells. These experiments were carried out in a reporter strain (called PD4251) expressing two different reporter proteins: nuclear GFP-LacZ and mitochondrial GFP. The micrographs show progeny of injected animals visualized by a fluorescence microscope. Panels A (young larva), B (adult), and C (adult body wall; high magnification) result from injection of a control RNA (ds-unc22A). Panels D–F show progeny of animals injected with ds-gfpG. Panels G–I demonstrate specificity. Animals are injected with ds-lacZL RNA, which should affect the nuclear but not the mitochondrial reporter construct. Panel H shows a typical adult, with nuclear GFP-LacZ lacking in almost all body-wall muscles but retained in vulval muscles. Scale bars are 20 μm.

FIGS. 3A–D show effects of double-stranded RNA corresponding to mex-3 on levels of the endogenous mRNA. Micrographs show in situ hybridization to embryos (dark stain). Panel A: Negative control showing lack of staining in the absence of hybridization probe. Panel B: Embryo from uninjected parent (normal pattern of endogenous mex-3 RNA[20]). Panel C: Embryo from a parent injected with purified mex-3B antisense RNA. These embryos and the parent animals retain the mex-3 mRNA, although levels may have been somewhat less than wild type. Panel D: Embryo from a parent injected with dsRNA corresponding to mex-3B; no mex-3 RNA was detected. Scale: each embryo is approximately 50 μm in length.

FIG. 4 shows inhibitory activity of unc-22A as a function of structure and concentration. The main graph indicates fractions in each behavioral class. Embryos in the uterus and already covered with an eggshell at the time of injection were not affected and, thus, are not included. Progeny cohort groups are labeled 1 for 0–6 hours, 2 for 6–15 hours, 3 for 15–27 hours, 4 for 27–41 hours, and 5 for 41–56 hours. The bottom-left diagram shows genetically derived relationship between unc-22 gene dosage and behavior based on analyses of unc-22 heterozygotes and polyploids[9,3].

FIGS. 5A–C show examples of genetic inhibition following ingestion by *C. elegans* of dsRNAs from expressing bacteria. Panel A: General strategy for production of dsRNA by cloning a segment of interest between flanking copies of the bacteriophage T7 promoter and transcribing both strands of the segment by transfecting a bacterial strain (BL21/DE3)[28] expressing the T7 polymerase gene from an inducible (Lac) promoter. Panel B: A GFP-expressing *C. elegans* strain, PD4251 (see FIG. 2), fed on a native bacterial host. Panel C: PD4251 animals reared on a diet of bacteria expressing dsRNA corresponding to the coding region for gfp.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method of producing sequence-specific inhibition of gene expression by introducing double-stranded RNA (dsRNA). A process is provided for inhibiting expression of a target gene in a cell. The process comprises introduction of RNA with partial or fully double-stranded character into the cell. Inhibition is sequence-specific in that a nucleotide sequence from a portion of the target gene is chosen to produce inhibitory RNA. We disclose that this process is (1) effective in producing inhibition of gene expression, (2) specific to the targeted gene, and (3) general in allowing inhibition of many different types of target gene.

The target gene may be a gene derived from the cell (i.e., a cellular gene), an endogenous gene (i.e., a cellular gene present in the genome), a transgene (i.e., a gene construct inserted at an ectopic site in the genome of the cell), or a gene from a pathogen which is capable of infecting an organism from which the cell is derived. Depending on the particular target gene and the dose of double stranded RNA material delivered, this process may provide partial or complete loss of function for the target gene. A reduction or loss of gene expression in at least 99% of targeted cells has been shown.

Inhibition of gene expression refers to the absence (or observable decrease) in the level of protein and/or mRNA product from a target gene. Specificity refers to the ability to inhibit the target gene without manifest effects on other genes of the cell. The consequences of inhibition can be confirmed by examination of the outward properties of the cell or organism (as presented below in the examples) or by biochemical techniques such as RNA solution hybridization, nuclease protection, Northern hybridization, reverse transcription, gene expression monitoring with a microarray, antibody binding, enzyme linked immunosorbent assay (ELISA), Western blotting, radioimmunoassay (RIA), other immunoassays and fluorescence activated cell analysis (FACS). For RNA-mediated inhibition in a cell line or whole

organism, gene expression is conveniently assayed by use of a reporter or drug resistance gene whose protein product is easily assayed. Such reporter genes include acetohydroxy-acid synthase (AHAS), alkaline phosphatase (AP), beta galactosidase (LacZ), beta glucoronidase (GUS), chloramphenicol acetyltransferase (CAT), green fluorescent protein (GFP), horseradish peroxidase (HRP), luciferase (Luc), nopaline synthase (NOS), octopine synthase (OCS), and derivatives thereof. Multiple selectable markers are available that confer resistance to ampicillin, bleomycin, chloramphenicol, gentamycin, hygromycin, kanamycin, lincomycin, methotrexate, phosphinothricin, puromycin, and tetracyclin.

Depending on the assay, quantitation of the amount of gene expression allows one to determine a degree of inhibition which is greater than 10%, 33%, 50%, 90%, 95% or 99% as compared to a cell not treated according to the present invention. Lower doses of injected material and longer times after administration of dsRNA may result in inhibition in a smaller fraction of cells (e.g., at least 10%, 20%, 50%, 75%, 90%, or 95% of targeted cells). Quantitation of gene expression in a cell may show similar amounts of inhibition at the level of accumulation of target mRNA or translation of target protein. As an example, the efficiency of inhibition may be determined by assessing the amount of gene product in the cell: mRNA may be detected with a hybridization probe having a nucleotide sequence outside the region used for the inhibitory double-stranded RNA, or translated polypeptide may be detected with an antibody raised against the polypeptide sequence of that region.

The RNA may comprise one or more strands of polymerized ribonucleotides. It may include modifications to either the phosphate-sugar backbone or the nucleoside. For example, the phosphodiester linkages of natural RNA may be modified to include at least one of a nitrogen or sulfur heteroatom. Modifications in RNA structure may be tailored to allow specific genetic inhibition while avoiding a general panic response in some organisms which is generated by dsRNA. Likewise, bases may be modified to block the activity of adenosine deaminase. RNA may be produced enzymatically or by partial/total organic synthesis, any modified ribonucleotide can be introduced by in vitro enzymatic or organic synthesis.

The double-stranded structure may be formed by a single self-complementary RNA strand or two complementary RNA strands. RNA duplex formation may be initiated either inside or outside the cell. The RNA may be introduced in an amount which allows delivery of at least one copy per cell. Higher doses (e.g., at least 5, 10, 100, 500 or 1000 copies per cell) of double-stranded material may yield more effective inhibition; lower doses may also be useful for specific applications. *Inhibition is sequence-specific in that nucleotide sequences corresponding to the duplex region of the RNA are targeted for genetic inhibition.*

RNA containing a nucleotide sequences identical to a portion of the target gene are preferred for inhibition. RNA sequences with insertions, deletions, and single point mutations relative to the target sequence have also been found to be effective for inhibition. Thus, sequence identity may optimized by sequence comparison and alignment algorithms known in the art (see Gribskov and Devereux, Sequence Analysis Primer, Stockton Press, 1991, and references cited therein) and calculating the percent difference between the nucleotide sequences by, for example, the Smith-Waterman algorithm as implemented in the BESTFIT software program using default parameters (e.g., University of Wisconsin Genetic Computing Group). Greater than 90% sequence identity, or even 100% sequence identity, between the inhibitory RNA and the portion of the target gene is preferred. Alternatively, the duplex region of the RNA may be defined functionally as a nucleotide sequence that is capable of hybridizing with a portion of the target gene transcript (e.g., 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA, 50° C. or 70° C. hybridization for 12–16 hours; followed by washing). The length of the identical nucleotide sequences may be at least 25, 50, 100, 200, 300 or 400 bases.

As disclosed herein, 100% sequence identity between the RNA and the target gene is not required to practice the present invention. Thus the invention has the advantage of being able to tolerate sequence variations that might be expected due to genetic mutation, strain polymorphism, or evolutionary divergence.

The cell with the target gene may be derived from or contained in any organism. The organism may a plant, animal, protozoan, bacterium, virus, or fungus. The plant may be a monocot, dicot or gymnosperm; the animal may be a vertebrate or invertebrate. Preferred microbes are those used in agriculture or by industry, and those that are pathogenic for plants or animals. Fungi include organisms in both the mold and yeast morphologies.

Plants include arabidopsis, field crops (e.g., alfalfa, barley, bean, corn, cotton, flax, pea, rape, rice, rye, safflower, sorghum, soybean, sunflower, tobacco, and wheat), vegetable crops (e.g., asparagus, beet, broccoli, cabbage, carrot, cauliflower, celery, cucumber, eggplant, lettuce, onion, pepper, potato, pumpkin, radish, spinach, squash, taro, tomato, and zucchini); fruit and nut crops (e.g., almond, apple, apricot, banana, blackberry, blueberry, cacao, cherry, coconut, cranberry, date, fajoa, filbert, grape, grapefruit, guava, kiwi, lemon, lime, mango, melon, nectarine, orange, papaya, passion fruit, peach, peanut, pear, pineapple, pistachio, plum, raspberry, strawberry, tangerine, walnut, and watermelon); and ornamentals (e.g., alder, ash, aspen, azalea, birch, boxwood, camellia, carnation, chrysanthemum, elm, fir, ivy, jasmine, juniper, oak, palm, poplar, pine, redwood, rhododendron, rose, and rubber).

Examples of vertebrate animals include fish, mammal, cattle, goat, pig, sheep, rodent, hamster, mouse, rat, primate, and human; invertebrate animals include nematodes, other worms, drosophila, and other insects. Representative genera of nematodes include those that infect animals (e.g., Ancylostoma, Ascaridia, Ascaris, Bunostomum, Caenorhabditis, Capillaria, Chabertia, Cooperia, Dictyocaulus, Haemonchus, Heterakis, Nematodirus, Oesophagostomum, Ostertagia, Oxyuris, Parascaris, Strongylus, Toxascaris, Trichuris, Trichostrongylus, Tfhchonema, Toxocara, Uncinaria) and those that infect plants (e.g., Bursaphalenchus, Criconemella, Diiylenchus, Ditylenchus, Globodera, Helicotylenchus, Heterodera, Longidorus, Melodoigyne, Nacobbus, Paratylenchus, Pratylenchus, Radopholus, Rotelynchus, Tylenchus, and Xiphinema). Representative orders of insects include Coleoptera, Diptera, Lepidoptera, and Homoptera.

The cell having the target gene may be from the germ line or somatic, totipotent or pluripotent, dividing or non-dividing, parenchyma or epithelium, immortalized or transformed, or the like. The cell may be a stem cell or a differentiated cell. Cell types that are differentiated include adipocytes, fibroblasts, myocytes, cardiomyocytes, endothelium, neurons, glia, blood cells, megakaryocytes, lymphocytes, macrophages, neutrophils, eosinophils, basophils, mast cells, leukocytes, granulocytes, keratinocytes, chondrocytes, osteoblasts, osteoclasts, hepatocytes, and cells of the endocrine or exocrine glands.

RNA may be synthesized either in vivo or in vitro. Endogenous RNA polymerase of the cell may mediate transcription in vivo, or cloned RNA polymerase can be used for transcription in vivo or in vitro. For transcription from a transgene in vivo or an expression construct a regulatory region (e.g., promoter, enhancer, silencer, splice donor and

acceptor, polyadenylation) may be used to transcribe the RNA strand (or strands). Inhibition may be targeted by specific transcription in an organ, tissue, or cell type; stimulation of an environmental condition (e.g., infection, stress, temperature, chemical inducers); and/or engineering transcription at a developmental stage or age. The RNA strands may or may not be polyadenylated; the RNA strands may or may not be capable of being translated into a polypeptide by a cell's translational apparatus. RNA may be chemically or enzymatically synthesized by manual or automated reactions. The RNA may be synthesized by a cellular RNA polymerase or a bacteriophage RNA polymerase (e.g., T3, T7, SP6). The use and production of an expression construct are known in the art (see also WO 97/32016; U.S. Pat. Nos. 5,593,874, 5,698,425, 5,712,135, 5,789,214, and 5,804,693; and the references cited therein). If synthesized chemically or by in vitro enzymatic synthesis, the RNA may be purified prior to introduction into the cell. For example, RNA can be purified from a mixture by extraction with a solvent or resin, precipitation, electrophoresis, chromatography, or a combination thereof. Alternatively the RNA may be used with no or a minimum of purification to avoid losses due to sample processing. The RNA may be dried for storage or dissolved in an aqueous solution. The solution may contain buffers or salts to promote annealing, and/or stabilization of the duplex strands.

RNA may be directly introduced into the cell (i.e., intracellularly); or introduced extracellularly into a cavity, interstitial space, into the circulation of an organism, introduced orally, or may be introduced by bathing an organism in a solution containing the RNA. Methods for oral introduction include direct mixing of the RNA with food of the organism, as well as engineered approaches in which a species that is used as food is engineered to express the RNA, then fed to the organism to be affected. For example, the RNA may be sprayed onto a plant or a plant may be genetically engineered to express the RNA in an amount sufficient to kill some or all of a pathogen known to infect the plant. Physical methods of introducing nucleic acids, for example, injection directly into the cell or extracellular injection into the organism, may also be used. We disclose herein that in *C. elegans*, double-stranded RNA introduced outside the cell inhibits gene expression. Vascular or extravascular circulation, the blood or lymph system, the phloem, the roots, and the cerebrospinal fluid are sites where the RNA may be introduced. (A transgenic organism that expresses RNA from a recombinant construct may be produced by introducing the construct into a zygote, an embryonic stem cell, or another multipotent cell derived from the appropriate organism.)

Physical methods of introducing nucleic acids include injection of a solution containing the RNA, bombardment by particles covered by the RNA, soaking the cell or organism in a solution of the RNA, or electroporation of cell membranes in the presence of the RNA. A viral construct packaged into a viral particle would accomplish both efficient introduction of an expression construct into the cell and transcription of RNA encoded by the expression construct. Other methods known in the art for introducing nucleic acids to cells may be used, such as lipid-mediated carrier transport, chemical-mediated transport, such as calcium phosphate, and the like. Thus the RNA may be introduced along with components that perform one or more of the following activities: enhance RNA uptake by the cell, promote annealing of the duplex strands, stabilize the annealed strands, or other-wise increase inhibition of the target gene.

The present invention may be used to introduce RNA into a cell for the treatment or prevention of disease. For example, dsRNA may be introduced into a cancerous cell or tumor and thereby inhibit gene expression of a gene required for maintenance of the carcinogenic/tumorigenic phenotype. To prevent a disease or other pathology, a target gene may be selected which is required for initiation or maintenance of the disease/pathology. Treatment would include amelioration of any symptom associated with the disease or clinical indication associated with the pathology.

A gene derived from any pathogen may be targeted for inhibition. For example, the gene could cause immunosuppression of the host directly or be essential for replication of the pathogen, transmission of the pathogen, or maintenance of the infection. The inhibitory RNA could be introduced in cells in vitro or ex vivo and then subsequently placed into an animal to affect therapy, or directly treated by in vivo administration. A method of gene therapy can be envisioned. For example, cells at risk for infection by a pathogen or already infected cells, particularly human immunodeficiency virus (HIV) infections, may be targeted for treatment by introduction of RNA according to the invention. The target gene might be a pathogen or host gene responsible for entry of a pathogen into its host, drug metabolism by the pathogen or host, replication or integration of the pathogen's genome, establishment or spread of an infection in the host, or assembly of the next generation of pathogen. Methods of *prophylaxis* (i.e., prevention or decreased risk of infection), as well as reduction in the frequency or severity of symptoms associated with infection, can be envisioned.

The present invention could be used for treatment or development of treatments for cancers of any type, including solid tumors and leukemias, including: apudoma, choristoma, branchioma, malignant carcinoid syndrome, carcinoid heart disease, carcinoma (e.g., Walker, basal cell, basosquamous, Brown-Pearce, ductal, Ehrlich tumor, in situ, Krebs 2, Merkel cell, mucinous, non-small cell lung, oat cell, papillary, scirrhous, bronchiolar, bronchogenic, squamous cell, and transitional cell), histiocytic disorders, leukemia (e.g., B cell, mixed cell, null cell, T cell, T-cell chronic, HTLV-II-associated, lymphocytic acute, lymphocytic chronic, mast cell, and myeloid), histiocytosis malignant, Hodgkin disease, immunoproliferative small, non-Hodgkin lymphoma, plasmacytoma, reticuloendotheliosis, melanoma, chondroblastoma, chondroma, chondrosarcoma, fibroma, fibrosarcoma, giant cell tumors, histiocytoma, lipoma, liposarcoma, mesothelioma, myxoma, myxosarcoma, osteoma, osteosarcoma, Ewing sarcoma, synovioma, adenofibroma, adenolymphoma, carcinosarcoma, chordoma, craniopharyngioma, dysgerminoma, hamartoma, mesenchymoma, mesonephroma, myosarcoma, ameloblastoma, cementoma, odontoma, teratoma, thymoma, trophoblastic tumor, adenocarcinoma, adenoma, cholangioma, cholesteatoma, cylindroma, cystadenocarcinoma, cystadenoma, granulosa cell tumor, gynandroblastoma, hepatoma, hidradenoma, islet cell tumor, Leydig cell tumor, papilloma, Sertoli cell tumor, theca cell tumor, leiomyoma, leiomyosarcoma, myoblastoma, myoma, myosarcoma, rhabdomyoma, rhabdomyosarcoma, ependymoma, ganglioneuroma, glioma, medulloblastoma, meningioma, neurilemmoma, neuroblastoma, neuroepithelioma, neurofibroma, neuroma, paraganglioma, paraganglioma nonchromaffin, angiokeratoma, angiolymphoid hyperplasia with eosinophilia, angioma sclerosing, angiomatosis, glomangioma, hemangioendothelioma, hemangioma, hemangiopericytoma, hemangiosarcoma, lymphangioma, lymphangiomyoma, lymphangiosarcoma, pinealoma, carcinosarcoma, chondrosarcoma, cystosarcoma phyllodes, fibrosarcoma, hemangiosarcoma, leiomyosarcoma, leukosarcoma, liposarcoma, lymphangiosarcoma, myosarcoma, myxosarcoma, ovarian carcinoma, rhabdomyosarcoma, sarcoma (e.g., Ewing, experimental, Kaposi, and mast cell), neoplasms (e.g., bone, breast, diges-

tive system, colorectal, liver, pancreatic, pituitary, testicular, orbital, head and neck, central nervous system, acoustic, pelvic, respiratory tract, and urogenital), neurofibromatosis, and cervical dysplasia, and for treatment of other conditions in which cells have become immortalized or transformed. The invention could be used in combination with other treatment modalities, such as chemotherapy, cryotherapy, hyperthermia, radiation therapy, and the like.

As disclosed herein, the present invention may is not limited to any type of target gene or nucleotide sequence. But the following classes of possible target genes are listed for illustrative purposes: developmental genes (e.g., adhesion molecules, cyclin kinase inhibitors, Wnt family members, Pax family members, Winged helix family members, Hox family members, cytokines/lymphokines and their receptors, growth/differentiation factors and their receptors, neurotransmitters and their receptors); oncogenes (e.g., ABL1, BCL1, BCL2, BCL6, CBFA2, CBL, CSF1R, ERBA, ERBB, EBRB2, ETS1, ETS1, ETV6, FGR, FOS, FYN, HCR, HRAS, JUN, KRAS, LCK, LYN, MDM2, MLL, MYB, MYC, MYCL1, MYCN, NRAS, PIM1, PML, RET, SRC, TAL1, TCL3, and YES); tumor suppressor genes (e.g., APC, BRCA1, BRCA2, MADH4, MCC, NF1, NF2, RB1, TP53, and WT1); and enzymes (e.g., ACC synthases and oxidases, ACP desaturases and hydroxylases, ADP-glucose pyrophorylases, ATPases, alcohol dehydrogenases, amylases, amyloglucosidases, catalases, cellulases, chalcone synthases, chitinases, cyclooxygenases, decarboxylases, dextrinases, DNA and RNA polymerases, galactosidases, glucanases, glucose oxidases, granule-bound starch synthases, GTPases, helicases, hemicellulases, integrases, inulinases, invertases, isomerases, kinases, lactases, lipases, lipoxygenases, lysozymes, nopaline synthases, octopine synthases, pectinesterases, peroxidases, phosphatases, phospholipases, phosphorylases, phytases, plant growth regulator synthases, polygalacturonases, proteinases and peptidases, pullanases, recombinases, reverse transcriptases, RUBISCOs, topoisomerases, and xylanases).

The present invention could comprise a method for producing plants with reduced susceptibility to climatic injury, susceptibility to insect damage, susceptibility to infection by a pathogen, or altered fruit ripening characteristics. The targeted gene may be an enzyme, a plant structural protein, a gene involved in pathogenesis, or an enzyme that is involved in the production of a non-proteinaceous part of the plant (i.e., a carbohydrate or lipid). If an expression construct is used to transcribe the RNA in a plant, transcription by a wound- or stress-inducible; tissue-specific (e.g., fruit, seed, anther, flower, leaf, root); or otherwise regulatable (e.g., infection, light, temperature, chemical) promoter may be used. By inhibiting enzymes at one or more points in a metabolic pathway or genes involved in pathogenesis, the effect may be enhanced: each activity will be affected and the effects may be magnified by targeting multiple different components. Metabolism may also be manipulated by inhibiting feedback control in the pathway or production of unwanted metabolic byproducts.

The present invention may be used to reduce crop destruction by other plant pathogens such as arachnids, insects, nematodes, protozoans, bacteria, or fungi. Some such plants and their pathogens are listed in *Index of plant Diseases in the United States* (U.S. Dept. of Agriculture Handbook No. 165, 1960); Distribution of Plant-Parasitic Nematode Species in North America (Society of Nematologists, 1985); and Fungi on Plants and Plant Products in the United States (American Phytopathological Society, 1989). Insects with reduced ability to damage crops or improved ability to prevent other destructive insects from damaging crops may be produced. Furthermore, some nematodes are vectors of plant pathogens, and may be attacked by other beneficial nematodes which have no effect on plants. Inhibition of target gene activity could be used to delay or prevent entry into a particular developmental step (e.g., metamorphosis), if plant disease was associated with a particular stage of the pathogen's life cycle. Interactions between pathogens may also be modified by the invention to limit crop damage. For example, the ability of beneficial nematodes to attack their harmful prey may be enhanced by inhibition of behavior-controlling nematode genes according to the invention.

Although pathogens cause disease, some of the microbes interact with their plant host in a beneficial manner. For example, some bacteria are involved in symbiotic relationships that fix nitrogen and some fungi produce phytohormones. Such beneficial interactions may be promoted by using the present invention to inhibit target gene activity in the plant and/or the microbe.

Another utility of the present invention could be a method of identifying gene function in an organism comprising the use of double-stranded RNA to inhibit the activity of a target gene of previously unknown function. Instead of the time consuming and laborious isolation of mutants by traditional genetic screening, functional genomics would envision determining the function of uncharacterized genes by employing the invention to reduce the amount and/or alter the timing of target gene activity. The invention could be used in determining potential targets for pharmaceutics, understanding normal and pathological events associated with development, determining signaling pathways responsible for postnatal development/aging, and the like. The increasing speed of acquiring nucleotide sequence information from genomic and expressed gene sources, including total sequences for the yeast, *D. melanogaster*, and *C. elegans* genomes, can be coupled with the invention to determine gene function in an organism (e.g., nematode). The preference of different organisms to use particular codons, searching sequence databases for related gene products, correlating the linkage map of genetic traits with the physical map from which the nucleotide sequences are derived, and artificial intelligence methods may be used to define putative open reading frames from the nucleotide sequences acquired in such sequencing projects.

A simple assay would be to inhibit gene expression according to the partial sequence available from an expressed sequence tag (EST). Functional alterations in growth, development, metabolism, disease resistance, or other biological processes would be indicative of the normal role of the EST's gene product.

The ease with which RNA can be introduced into an intact cell/organism containing the target gene allows the present invention to be used in high throughput screening (HTS). For example, duplex RNA can be produced by an amplification reaction using primers flanking the inserts of any gene library derived from the target cell/organism. Inserts may be derived from genomic DNA or mRNA (e.g., cDNA and cRNA). Individual clones from the library can be replicated and then isolated in separate reactions, but preferably the library is maintained in individual reaction vessels (e.g., a 96-well microtiter plate) to minimize the number of steps required to practice the invention and to allow automation of the process. Solutions containing duplex RNAs that are capable of inhibiting the different expressed genes can be placed into individual wells positioned on a microtiter plate as an ordered array, and intact cells/organisms in each well can be assayed for any changes or modifications in behavior or development due to inhibition of target gene activity. The amplified RNA can be fed directly to, injected into, the cell/organism containing the target gene. Alternatively, the duplex RNA can be produced by in vivo or in vitro transcription from an expression construct used to produce the library. The construct can be replicated as individual clones

of the library and transcribed to produce the RNA; each clone can then be fed to, or injected into, the cell/organism containing the target gene. The function of the target gene can be assayed from the effects it has on the cell/organism when gene activity is inhibited. This screening could be amenable to small subjects that can be processed in large number, for example: arabidopsis, bacteria, drosophila, fungi, nematodes, viruses, zebrafish, and tissue culture cells derived from mammals.

A nematode or other organism that produces a colorimetric, fluorogenic, or luminescent signal in response to a regulated promoter (e.g., transfected with a reporter gene construct) can be assayed in an HTS format to identify DNA-binding proteins that regulate the promoter. In the assay's simplest form, inhibition of a negative regulator results in an increase of the signal and inhibition of a positive regulator results in a decrease of the signal.

If a characteristic of an organism is determined to be genetically linked to a polymorphism through RFLP or QTL analysis, the present invention can be used to gain insight regarding whether that genetic polymorphism might be directly responsible for the characteristic. For example, a fragment defining the genetic polymorphism or sequences in the vicinity of such a genetic polymorphism can be amplified to produce an RNA, the duplex RNA can be introduced to the organism, and whether an alteration in the characteristic is correlated with inhibition can be determined. Of course, there may be trivial explanations for negative results with this type of assay, for example: inhibition of the target gene causes lethality, inhibition of the target gene may not result in any observable alteration, the fragment contains nucleotide sequences that are not capable of inhibiting the target gene, or the target gene's activity is redundant.

The present invention may be useful in allowing the inhibition of essential genes. Such genes may be required for cell or organism viability at only particular stages of development or cellular compartments. The functional equivalent of conditional mutations may be produced by inhibiting activity of the target gene when or where it is not required for viability. The invention allows addition of RNA at specific times of development and locations in the organism without introducing permanent mutations into the target genome.

If alternative splicing produced a family of transcripts that were distinguished by usage of characteristic exons, the present invention can target inhibition through the appropriate exons to specifically inhibit or to distinguish among the functions of family members. For example, a hormone that contained an alternatively spliced transmembrane domain may be expressed in both membrane bound and secreted forms. Instead of isolating a nonsense mutation that terminates translation before the transmembrane domain, the functional consequences of having only secreted hormone can be determined according to the invention by targeting the exon containing the transmembrane domain and thereby inhibiting expression of membrane-bound hormone.

The present invention may be used alone or as a component of a kit having at least one of the reagents necessary to carry out the in vitro or in vivo introduction of RNA to test samples or subjects. Preferred components are the dsRNA and a vehicle that promotes introduction of the dsRNA. Such a kit may also include instructions to allow a user of the kit to practice the invention.

Pesticides may include the RNA molecule itself, an expression construct capable of expressing the RNA, or organisms transfected with the expression construct. The pesticide of the present invention may serve as an arachnicide, insecticide, nematicide, viricide, bactericide, and/or fungicide. For example, plant parts that are accessible above ground (e.g., flowers, fruits, buds, leaves, seeds, shoots, bark, stems) may be sprayed with pesticide, the soil may be soaked with pesticide to access plant parts growing beneath ground level, or the pest may be contacted with pesticide directly. If pests interact with each other, the RNA may be transmitted between them. Alternatively, if inhibition of the target gene results in a beneficial effect on plant growth or development, the aforementioned RNA, expression construct, or transfected organism may be considered a nutritional agent. In either case, genetic engineering of the plant is not required to achieve the objectives of the invention.

Alternatively, an organism may be engineered to produce dsRNA which produces commercially or medically beneficial results, for example, resistance to a pathogen or its pathogenic effects, improved growth, or novel developmental patterns.

Used as either an pesticide or nutrient, a formulation of the present invention may be delivered to the end user in dry or liquid form: for example, as a dust, granulate, emulsion, paste, solution, concentrate, suspension, or encapsulation. Instructions for safe and effective use may also be provided with the formulation. The formulation might be used directly, but concentrates would require dilution by mixing with an extender provided by the formulator or the end user. Similarly, an emulsion, paste, or suspension may require the end user to perform certain preparation steps before application. The formulation may include a combination of chemical additives known in the art such as solid carriers, minerals, solvents, dispersants, surfactants, emulsifiers, tackifiers, binders, and other adjuvants. Preservatives and stabilizers may also be added to the formulation to facilitate storage. The crop area or plant may also be treated simultaneously or separately with other pesticides or fertilizers. Methods of application include dusting, scattering or pouring, soaking, spraying, atomizing, and coating. The precise physical form and chemical composition of the formulation, and its method of application, would be chosen to promote the objectives of the invention and in accordance with prevailing circumstances. Expression constructs and transfected hosts capable of replication may also promote the persistence and/or spread of the formulation.

Description of the dsRNA Inhibition Phenomenon in *C. elegans*

The operation of the present invention was shown in the model genetic organism *Caenorhabditis elegans*.

Introduction of RNA into cells had been seen in certain biological systems to interfere with function of an endogenous gene [1,2]. Many such effects were believed to result from a simple antisense mechanism dependent on hybridization between injected single-stranded RNA and endogenous transcripts. In other cases, a more complex mechanism had been suggested. One instance of an RNA-mediated mechanism was RNA interference (RNAi) phenomenon in the nematode *C. elegans* (RNAi) had been used in a variety of studies to manipulate gene expression[3,4].

Despite the usefulness of RNAi in C. elegans, many features had been difficult to explain. Also, the lack of a clear understanding of the critical requirements for interfering RNA led to a sporadic record of failure and partial success in attempts to extend RNAi beyond the earliest stages following injection. A statement frequently made in the literature was that sense and antisense RNA preparations are each sufficient to cause interference[3,4]. The only precedent for such a situation was in plants where the process of cosuppression had a similar history of usefulness in certain cases, failure in others, and no ability to design interference protocols with a high chance of success. Working with *C. elegans*, we discovered an RNA structure that would give

effective and uniform genetic inhibition. The prior art did not teach or suggest that RNA structure was a critical feature for inhibition of gene expression. Indeed the ability of crude sense and antisense preparations to produce interference[3,4] had been taken as an indication that RNA structure was not a critical factor. Instead, the extensive plant literature and much of the ongoing research in *C. elegans* was focused on the possibility that detailed features of the target gene sequence or its chromosomal locale was the critical feature for interfering with gene expression.

The inventors carefully purified sense or antisense RNA for unc-22 and tested each for gene-specific inhibition. While the crude sense and antisense preparations had strong interfering activity, it was found that the purified sense and antisense RNAs had only marginal inhibitory activity. This was unexpected because many techniques in molecular biology are based on the assumption that RNA produced with specific in vitro promoters (e.g., T3 or T7 RNA polymerase), or with characterized promoters in vivo, is produced predominantly from a single strand. The inventors had carried out purification of these crude preparations to investigate whether a small fraction of the RNA had an unusual structure which might be responsible for the observed genetic inhibition. To rigorously test whether double-stranded character might contribute to genetic inhibition, the inventors carried out additional purification of single-stranded RNAs and compared inhibitory activities of individual strands with that of the double-stranded hybrid.

The following examples are meant to be illustrative of the present invention; however, the practice of the invention is not limited or restricted in any way by them.

Analysis of RNA-Mediated Inhibition of *C. elegans* Genes

The unc-22 gene was chosen for initial comparisons of activity as a result of previous genetic analysis that yields a semi-quantitative comparison between unc-22 gene activity and the movement phenotypes of animals[3,8]: decreases in activity produce an increasingly severe twitching phenotype, while complete loss of function results in the additional appearance of muscle structural defects and impaired motility. unc-22 encodes an abundant but non-essential myofilament protein[7-9]. unc-22 mRNA is present at several thousand copies per striated muscle cell[3].

Purified antisense and sense RNAs covering a 742 nt segment of unc-22 had only marginal inhibitory activity, requiring a very high dose of injected RNA for any observable effect (FIG. 4). By contrast, a sense+antisense mixture produced a highly effective inhibition of endogenous gene activity (FIG. 4). The mixture was at least two orders of magnitude more effective than either single strand in inhibiting gene expression. The lowest dose of the sense+antisense mixture tested, approximately 60,000 molecules of each strand per adult, led to twitching phenotypes in an average of 100 progeny. unc-22 expression begins in embryos with approximately 500 cells. At this point, the original injected material would be diluted to at most a few molecules per cell.

The potent inhibitory activity of the sense+antisense mixture could reflect formation of double-stranded RNA (dsRNA), or conceivably some alternate synergy between the strands. Electrophoretic analysis indicated that the injected material was predominantly double stranded. The dsRNA was gel purified from the annealed mixture and found to retain potent inhibitory activity. Although annealing prior to injection was compatible with inhibition, it was not necessary. Mixing of sense and antisense RNAs in low salt (under conditions of minimal dsRNA formation), or rapid sequential injection of sense and antisense strands, were sufficient to allow complete inhibition. A long interval (>1 hour) between sequential injections of sense and antisense RNA resulted in a dramatic decrease in inhibitory activity. This suggests that injected single strands may be degraded or otherwise rendered inaccessible in the absence of the complementary strand.

An issue of specificity arises when considering known cellular responses to dsRNA. Some organisms have a dsRNA-dependent protein kinase that activates a panic response mechanism[10]. Conceivably, the inventive sense+antisense synergy could reflect a non-specific potentiation of antisense effects by such a panic mechanism. This was not found to be the case: co-injection of dsRNA segments unrelated to unc-22 did not potentiate the ability of unc-22 single strands to mediate inhibition. Also investigated was whether double-stranded structure could potentiate inhibitory activity when placed in cis to a single-stranded segment. No such potentiation was seen; unrelated double-stranded sequences located 5' or 3' of a single-stranded unc-22 segment did not stimulate inhibition. Thus potentiation of gene-specific inhibition was observed only when dsRNA sequences exist within the region of homology with the target gene.

The phenotype produced by unc-22 dsRNA was specific. Progeny of injected animals exhibited behavior indistinguishable from characteristic unc-22 loss of function mutants. Target-specificity of dsRNA effects using three additional genes with well characterized phenotypes (FIG. 1 and Table 1). unc-54 encodes a body wall muscle myosin heavy chain isoform required for full muscle contraction[7,11,12], fem-1 encodes an ankyrin-repeat containing protein required in hermaphrodites for sperm production[13,14], and hlh-1 encodes a *C. elegans* homolog of the myoD family required for proper body shape and motility[15,16]. For each of these genes, injection of dsRNA produced progeny broods exhibiting the known null mutant phenotype, while the purified single strands produced no significant reduction in gene expression. With one exception, all of the phenotypic consequences of dsRNA injection were those expected from inhibition of the corresponding gene. The exception (segment unc54C, which led to an embryonic and larval arrest phenotype not seen with unc-54 null mutants) was illustrative. This segment covers the highly conserved myosin motor domain, and might have been expected to inhibit the activity of other highly related myosin heavy chain genes[17]. This interpretation would support uses of the present invention in which nucleotide sequence comparison of dsRNA and target gene show less than 100% identity. The unc54C segment has been unique in our overall experience to date: effects of 18 other dsRNA segments have all been limited to those expected from characterized null mutants.

The strong phenotypes seen following dsRNA injection are indicative of inhibitory effects occurring in a high fraction of cells. The unc-54 and hlh-1 muscle phenotypes, in particular, are known to result from a large number of defective muscle cells[11,16]. To examine inhibitory effects of dsRNA on a cellular level, a transgenic line expressing two different GFP-derived fluorescent reporter proteins in body muscle was used. Injection of dsRNA directed to gfp produced dramatic decreases in the fraction of fluorescent cells (FIG. 2). Both reporter proteins were absent from the negative cells, while the few positive cells generally expressed both GFP forms.

The pattern of mosaicism observed with gfp inhibition was not random. At low doses of dsRNA, the inventors saw frequent inhibition in the embryonically-derived muscle cells present when the animal hatched. The inhibitory effect in these differentiated cells persisted through larval growth: these cells produced little or no additional GFP as the affected animals grew. The 14 postembryonically-derived

striated muscles are born during early larval stages and were more resistant to inhibition. These cells have come through additional divisions (13–14 versus 8–9 for embryonic muscles[18,19]). At high concentrations of gfp dsRNA, inhibition was noted in virtually all striated bodywall muscles, with occasional single escaping cells including cells born in embryonic or postembryonic stages. The nonstriated vulval muscles, born during late larval development, appeared resistant to genetic inhibition at all tested concentrations of injected RNA. The latter result is important for evaluating the use of the present invention in other systems. First, it indicates that failure in one set of cells from an organism does not necessarily indicate complete non-applicability of the invention to that organism. Second, it is important to realize that not all tissues in the organism need to be affected for the invention to be used in an organism. This may serve as an advantage in some situations.

A few observations serve to clarify the nature of possible targets and mechanisms for RNA-mediated genetic inhibition in *C. elegans:*

First, dsRNA segments corresponding to a variety of intron and promoter sequences did not produce detectable inhibition (Table 1). Although consistent with possible inhibition at a post-transcriptional level, these experiments do not rule out inhibition at the level of the gene.

Second, dsRNA injection produces a dramatic decrease in the level of the endogenous mRNA transcript (FIG. 3). Here, a mex-3 transcript that is abundant in the gonad and early embryos[20] was targeted where straightforward in situ hybridization can be performed. No endogenous mex-3 mRNA was observed in animals injected with a dsRNA segment derived from mex-3 (FIG. 3D), but injection of purified mex-3 antisense RNA resulted in animals that retained substantial endogenous mRNA levels (FIG. 3C).

Third, dsRNA-mediated inhibition showed a surprising ability to cross cellular boundaries. Injection of dsRNA for unc-22, gfp, or lacZ into the body cavity of the head or tail produced a specific and robust inhibition of gene expression in the progeny brood (Table 2). Inhibition was seen in the progeny of both gonad arms, ruling out a transient "nicking" of the gonad in these injections. dsRNA injected into body cavity or gonad of young adults also produced gene-specific inhibition in somatic tissues of the injected animal (Table 2).

Table 3 shows that *C. elegans* can respond in a gene-specific manner to dsRNA encountered in the environment. Bacteria are a natural food source for *C. elegans*. The bacteria are ingested, ground in the animal's pharynx, and the bacterial contents taken up in the gut. The results show that *E. coli* bacteria expressing dsRNAs can confer specific inhibitory effects on *C. elegans* nematode larvae that feed on them.

Three *C. elegans* genes were analyzed. For each gene, corresponding dsRNA was expressed in *E. coli* by inserting a segment of the coding region into a plasmid construct designed for bidirectional transcription by bacteriophage T7 RNA polymerase. The dsRNA segments used for these experiments were the same as those used in previous microinjection experiments (see FIG. 1). The effects resulting from feeding these bacteria to *C. elegans* were compared to the effects achieved by microinjecting animals with dsRNA.

The *C. elegans* gene unc-22 encodes an abundant muscle filament protein. unc-22 null mutations produce a characteristic and uniform twitching phenotype in which the animals can sustain only transient muscle contraction. When wild-type animals were fed bacteria expressing a dsRNA segment from unc-22, a high fraction (85%) exhibited a weak but still distinct twitching phenotype characteristic of partial loss of function for the unc-22 gene. The *C. elegans* fem-1 gene encodes a late component of the sex determination pathway. Null mutations prevent the production of sperm and lead euploid (XX) animals to develop as females, while wild type XX animals develop as hermaphrodites. When wild-type animals were fed bacteria expressing dsRNA corresponding to fem-1, a fraction (43%) exhibit a sperm-less (female) phenotype and were sterile. Finally, the ability to inhibit gene expression of a transgene target was assessed. When animals carrying a gfp transgene were fed bacteria expressing dsRNA corresponding to the gfp reporter, an obvious decrease in the overall level of GFP fluorescence was observed, again in approximately 12% of the population (see FIG. 5, panels B and C).

The effects of these ingested RNAs were specific. Bacteria carrying different dsRNAs from fem-1 and gfp produced no twitching, dsRNAs from unc-22 and fem-1 did not reduce gfp expression, and dsRNAs from gfp and unc-22 did not produce females. These inhibitory effects were apparently mediated by dsRNA: bacteria expressing only the sense or antisense strand for either gfp or unc-22 caused no evident phenotypic effects on their *C. elegans* predators.

Table 4 shows the effects of bathing *C. elegans* in a solution containing dsRNA. Larvae were bathed for 24 hours in solutions of the indicated dsRNAs (1 mg/ml), then allowed to recover in normal media and allowed to grow under standard conditions for two days. The unc-22 dsRNA was segment ds-unc22A from FIG. 1. pos-1 and sqt-3 dsRNAs were from the full length cDNA clones. pos-1 encodes an essential maternally provided component required early in embyogenesis. Mutations removing pos-1 activity have an early embryonic arrest characteristic of skn-like mutations[29,30]. Cloning and activity patterns for sqt-3 have been described[31]. *C. elegans* sqt-3 mutants have mutations in the col-1 collagen gene[31]. Phenotypes of affected animals are noted. Incidences of clear phenotypic effects in these experiments were 5–10% for unc-22, 50% for pos-1, and 5% for sqt-3. These are frequencies of unambiguous phenocopies; other treated animals may have had marginal defects corresponding to the target gene that were not observable. Each treatment was fully gene-specific in that unc-22 dsRNA produced only Unc-22 phenotypes, pos-1 dsRNA produced only Pos-1 phenotypes, and sqt-3 dsRNA produced only Sqt-3 phenotypes.

Some of the results described herein were published after the filing of our provisional application. Those publications and a review can be cited as Fire, A., et al. Nature, 391, 806–811, 1998; Timmons, L. & Fire, A. Nature, 395, 854, 1998; and Montgomery, M. K. & Fire, A. Trends in Genetics, 14, 255–258, 1998.

The effects described herein significantly augment available tools for studying gene function in *C. elegans* and other organisms. In particular, functional analysis should now be possible for a large number of interesting coding regions[21] for which no specific function have been defined. Several of these observations show the properties of dsRNA that may affect the design of processes for inhibition of gene expression. For example, one case was observed in which a nucleotide sequence shared between several myosin genes may inhibit gene expression of several members of a related gene family.

Methods of RNA Synthesis and Microinjection

RNA was synthesized from phagemid clones with T3 and T7 RNA polymerase[6], followed by template removal with two sequential DNase treatments. In cases where sense, antisense, and mixed RNA populations were to be compared, RNAs were further purified by electrophoresis on low-gelling-temperature agarose. Gel-purified products appeared to lack many of the minor bands seen in the original "sense" and "antisense" preparations. Nonetheless,

RNA species accounting for less than 10% of purified RNA preparations would not have been observed. Without gel purification, the "sense" and "antisense" preparations produced significant inhibition. This inhibitory activity was reduced or eliminated upon gel purification. By contrast, sense+antisense mixtures of gel purified and non-gel-purified RNA preparations produced identical effects.

Following a short (5 minute) treatment at 68° C. to remove secondary structure, sense+antisense annealing was carried out in injection buffer[27] at 37° C. for 10–30 minutes. Formation of predominantly double stranded material was confirmed by testing migration on a standard (non-denaturing) agarose gel: for each RNA pair, gel mobility was shifted to that expected for double-stranded RNA of the appropriate length. Co-incubation of the two strands in a low-salt buffer (5 mM Tris-HCl pH 7.5, 0.5 mM EDTA) was insufficient for visible formation of double-stranded RNA in vitro. Non-annealed sense+antisense RNAs for unc22B and gfpG were tested for inhibitory effect and found to be much more active than the individual single strands, but 2–4 fold less active than equivalent pre-annealed preparations.

After pre-annealing of the single strands for unc22A, the single electrophoretic species corresponding in size to that expected for dsRNA was purified using two rounds of gel electrophoresis. This material retained a high degree of inhibitory activity.

Except where noted, injection mixes were constructed so animals would receive an average of 0.5×10^6 to 1.0×10^6 molecules of RNA. For comparisons of sense, antisense, and dsRNA activities, injections were compared with equal masses of RNA (i.e., dsRNA at half the molar concentration of the single strands). Numbers of molecules injected per adult are given as rough approximations based on concentration of RNA in the injected material (estimated from ethidium bromide staining) and injection volume (estimated from visible displacement at the site of injection). A variability of several-fold in injection volume between individual animals is possible; however, such variability would not affect any of the conclusions drawn herein.

Methods for Analysis of Phenotypes

Inhibition of endogenous genes was generally assayed in a wild type genetic background (N2). Features analyzed included movement, feeding, hatching, body shape, sexual identity, and fertility. Inhibition with gfp[27] and lacZ activity was assessed using strain PD4251. This strain is a stable transgenic strain containing an integrated array (ccls4251) made up of three plasmids: pSAK4 (myo-3 promoter driving mitochondrially targeted GFP), pSAK2 (myo-3 promoter driving a nuclear targeted GFP-LacZ fusion), and a dpy-20 subclone[26] as a selectable marker. This strain produces GFP in all body muscles, with a combination of mitochondrial and nuclear localization. The two distinct compartments are easily distinguished in these cells, allowing a facile distinction between cells expressing both, either, or neither of the original GFP constructs.

Gonadal injection was performed by inserting the microinjection needle into the gonadal syncitium of adults and expelling 20–100 pl of solution (see Reference 25). Body cavity injections followed a similar procedure, with needle insertion into regions of the head and tail beyond the positions of the two gonad arms. Injection into the cytoplasm of intestinal cells was another effective means of RNA delivery, and may be the least disruptive to the animal. After recovery and transfer to standard solid media, injected animals were transferred to fresh culture plates at 16 hour intervals. This yields a series of semi-synchronous cohorts in which it was straightforward to identify phenotypic differences. A characteristic temporal pattern of phenotypic severity is observed among progeny. First, there is a short "clearance" interval in which unaffected progeny are produced. These include impermeable fertilized eggs present at the time of injection. After the clearance period, individuals are produced which show the inhibitory phenotype. After injected animals have produced eggs for several days, gonads can in some cases "revert" to produce incompletely affected or phenotypically normal progeny.

Additional Description of the Results

FIG. 1 shows genes used to study RNA-mediated genetic inhibition in *C. elegans*. Intron-exon structure for genes used to test RNA-mediated inhibition are shown (exons: filled boxes; introns: open boxes; 5' and 3' untranslated regions: shaded; sequence references are as follows: unc-22[9], unc-54[12], fem-1[14], and hlh-1[15]). These genes were chosen based on: (1) a defined molecular structure, (2) classical genetic data showing the nature of the null phenotype. Each segment tested for inhibitory effects is designated with the name of the gene followed by a single letter (e.g., unc22C). Segments derived from genomic DNA are shown above the gene, segments derived from cDNA are shown below the gene. The consequences of injecting double-stranded RNA segments for each of these genes is described in Table 1. dsRNA sequences from the coding region of each gene produced a phenotype resembling the null phenotype for that gene.

The effects of inhibitory RNA were analyzed in individual cells (FIG. 2, panels A–H). These experiments were carried out in a reporter strain (called PD4251) expressing two different reporter proteins: nuclear GFP-LacZ and mitochondrial GFP, both expressed in body muscle. The fluorescent nature of these reporter proteins allowed us to examine individual cells under the fluorescence microscope to determine the extent and generality of the observed inhibition of gene. ds-unc22A RNA was injected as a negative control. GFP expression in progeny of these injected animals was not affected. The GFP patterns of these progeny appear identical to the parent strain, with prominent fluorescence in nuclei (the nuclear localized GFP-LacZ) and mitochondria (the mitochondrially targeted GFP) in young larva (FIG. 2A), adult (FIG. 2B), and adult body wall at high magnification (FIG. 2C).

In contrast, the progeny of animals injected with ds-gfpG RNA are affected (FIGS. 2D–F). Observable GFP fluorescence is completely absent in over 95% of the cells. Few active cells were seen in larvae (FIG. 2D shows a larva with one active cell; uninjected controls show GFP activity in all 81 body wall muscle cells). Inhibition was not effective in all tissues: the entire vulval musculature expressed active GFP in an adult animal (FIG. 2E). Rare GFP positive body wall muscle cells were also seen in adult animals (two active cells are shown in FIG. 2F). Inhibition was target specific (FIGS. 2G–I). Animals were injected with ds-lacZL RNA, which should affect the nuclear but not the mitochondrial reporter construct. In the animals derived from this injection, mitochondrial-targeted GFP appeared unaffected while the nuclear-targeted GFP-LacZ was absent from almost all cells (larva in FIG. 2G). A typical adult lacked nuclear GFP-LacZ in almost all body-wall muscles but retained activity in vulval muscles (FIG. 2H). Scale bars in FIG. 2 are 20 μm.

The effects of double-stranded RNA corresponding to mex-3 on levels of the endogenous mRNA was shown by in situ hybridization to embryos (FIG. 3, panels A–D). The 1262 nt mex-3 cDNA clone[20] was divided into two segments, mex-3A and mex3B with a short (325 nt) overlap. Similar results were obtained in experiments with no overlap between inhibiting and probe segments. mex-3B antisense or dsRNA was injected into the gonads of adult animals, which were maintained under standard culture conditions for 24 hours before fixation and in situ hybridization (see

Reference 5). The mex-3B dsRNA produced 100% embryonic arrest, while >90% of embryos from the antisense injections hatched. Antisense probes corresponding to mex-3A were used to assay distribution of the endogenous mex-3 mRNA (dark stain). Four-cell stage embryos were assayed; similar results were observed from the 1 to 8 cell stage and in the germline of injected adults. The negative control (the absence of hybridization probe) showed a lack of staining (FIG. 3A). Embryos from uninjected parents showed a normal pattern of endogenous mex-3 RNA (FIG. 3B). The observed pattern of mex-3 RNA was as previously described in Reference 20. Injection of purified mex-3B antisense RNA produced at most a modest effect: the resulting embryos retained mex-3 mRNA, although levels may have been somewhat less than wild type (FIG. 3C). In contrast, no mex-3 RNA was detected in embryos from parents injected with dsRNA corresponding to mex-3 (FIG. 3D). The scale of FIG. 3 is such that each embryo is approximately 50 μm in length.

Gene-specific inhibitory activity by unc-22A RNA was measured as a function of RNA structure and concentration (FIG. 4). Purified antisense and sense RNA from unc22A were injected individually or as an annealed mixture. "Control" was an unrelated dsRNA (gfpG). Injected animals were transferred to fresh culture plates 6 hours (columns labeled 1), 15 hours (columns labeled 2), 27 hours (columns labeled 3), 41 hours (columns labeled 4), and 56 hours (columns labeled 5) after injection. Progeny grown to adulthood were scored for movement in their growth environment, then examined in 0.5 mM levamisole. The main graph indicates fractions in each behavioral class. Embryos in the uterus and already covered with an eggshell at the time of injection were not affected and, thus, are not included in the graph. The bottom-left diagram shows the genetically derived relationship between unc-22 gene dosage and behavior based on analyses of unc-22 heterozygotes and polyploids[28, 3].

FIGS. 5A–C show a process and examples of genetic inhibition following ingestion by *C. elegans* of dsRNAs from expressing bacteria. A general strategy for production of dsRNA is to clone segments of interest between flanking copies of the bacteriophage T7 promoter into a bacterial plasmid construct (FIG. 5A). A bacterial strain (BL21/DE3)[28] expressing the T7 polymerase gene from an inducible (Lac) promoter was used as a host. A nuclease-resistant dsRNA was detected in lysates of transfected bacteria. Comparable inhibition results were obtained with the two bacterial expression systems. A GFP-expressing *C. elegans* strain, PD4251 (see FIG. 2), was fed on a native bacterial host. These animals show a uniformly high level of GFP fluorescence in body muscles (FIG. 5B). PD4251 animals were also reared on a diet of bacteria expressing dsRNA corresponding to the coding region for gfp. Under the conditions of this experiment, 12% of these animals showed dramatic decreases in GFP (FIG. 5C). As an alternative strategy, single copies of the T7 promoter were used to drive expression of an inverted-duplication for a segment of the target gene, either unc-22 or gfp. This was comparably effective.

All references (e.g., books, articles, applications, and patents) cited in this specification are indicative of the level of skill in the art and their disclosures are incorporated herein in their entirety.

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TABLE 1

Effects of sense, antisense, and mixed RNAs on progeny of injected animals.

Gene and Segment		Size	Injected RNA	F1 Phenotype
unc-22				unc-22 null mutants: strong twitchers[7,8]
unc22A[a]	exon 21–22	742	sense	wild type
			antisense	wild type
			sense + antisense	strong twitchers (100%)
unc22B	exon27	1033	sense	wild type
			antisense	wild type

TABLE 1-continued

Gene and Segment		Size	Effects of sense, antisense, and mixed RNAs on progeny of injected animals. Injected RNA	F1 Phenotype
			sense + antisense	strong twitchers (100%)
unc22C	exon 21–22[b]	785	sense + antisense	strong twitchers (100%)
fem-1				fem-1 null mutants: female (no sperm)[13]
fem1A	exon 10[c]	531	sense	hermaphrodite (98%)
			antisense	hermaphrodite (>98%)
			sense + antisense	female (72%)
fem1B	intron 8	556	sense + antisense	hermaphrodite (>98%)
unc-54				unc-54 null mutants: paralyzed[7,11]
unc54A	exon 6	576	sense	wild type (100%)
			antisense	wild type (100%)
			sense + antisense	paralyzed (100%)[d]
unc54B	exon 6	651	sense	wild type (100%)
			antisense	wild type (100%)
			sense + antisense	paralyzed (100%)[d]
unc54C	exon 1–5	1015	sense + antisense	arrested embryos and larvae (100%)
unc54D	promoter	567	sense + antisense	wild type (100%)
unc54E	intron 1	369	sense + antisense	wild type (100%)
unc54F	intron 3	386	sense + antisense	wild type (100%)
hlh-1				hlh-1 null mutants: lumpy-dumpy larvae[16]
hlh1A	exons 1–6	1033	sense	wild type (<2% lpy-dpy)
			antisense	wild type (<2% lpy-dpy)
			sense + antisense	lpy-dpy larvae (>90%)[e]
hlh1B	exons 1–2	438	sense + antisense	lpy-dpy larvae (>80%)[e]
hlh1C	exons 4–6	299	sense + antisense	lpy-dpy larvae (>80%)[e]
hlh1D	intron 1	697	sense + antisense	wild type (<2% lpy-dpy)
myo-3 driven GFP transgenes[f]				makes nuclear GFP in body muscle
myo-3::NLS::gfp::lacZ				
gfpG	exons 2–5	730	sense	nuclear GFP-LacZ pattern of parent strain
			antisense	nuclear GFP-LacZ pattern of parent strain
			sense + antisense	nuclear GFP-LacZ absent in 98% of cells
lacZL	exon 12–14	830	sense + antisense	nuclear GFP-LacZ absent in >95% of cells
myo-3::MtLS::gfp				makes mitochondrial GFP in body muscle
gfpG	exons 2–5	730	sense	mitochondrial GFP pattern of parent strain
			antisense	mitochondrial GFP pattern of parent strain
			sense + antisense	mitochondrial GFP absent in 98% of cells
lacZL	exon 12–14	830	sense + antisense	mitochondrial GFP pattern of parent strain

Legend of Table 1

Each RNA was injected into 6–10 adult hermaphrodites ($0.5–1\times10^6$ molecules into each gonad arm). After 4–6 hours (to clear pre-fertilized eggs from the uterus) injected animals were transferred and eggs collected for 20–22 hours. Progeny phenotypes were scored upon hatching and subsequently at 12–24 hour intervals.

a: To obtain a semi-quantitative assessment of the relationship between RNA dose and phenotypic response, we injected each unc22A RNA preparation at a series of different concentrations. At the highest dose tested (3.6×10^6 molecules per gonad), the individual sense and antisense unc22A preparations produced some visible twitching (1% and 11% of progeny respectively). Comparable doses of ds-unc22A RNA produced visible twitching in all progeny, while a 120-fold lower dose of ds-unc22A RNA produced visible twitching in 30% of progeny.

b: unc22C also carries the intervening intron (43 nt).

c: fem1A also carries a portion (131 nt) of intron 10.

d: Animals in the first affected broods (and at 4–24 hours after injection) showed movement defects indistinguishable from those of null mutants in unc-54. A variable fraction of these animals (25–75%) failed to lay eggs (another phenotype of unc-54 null mutants), while the remainder of the paralyzed animals were egg-laying positive. This may indicate partial inhibition of unc-54 activity in vulval muscles. Animals from later broods frequently exhibit a distinct partial loss-of-function phenotype, with contractility in a subset of body wall muscles.

e: Phenotypes of hlh-1 inhibitory RNA include arrested embryos and partially elongated L1 larvae (the hlh-1 null phenotype) seen in virtually all progeny from injection of ds-hlh1A and about half of the affected animals from ds-hlh1B and ds-hlh1C) and a set of less severe defects (seen with the remainder of the animals from ds-hlh1B and ds-hlh1C). The less severe phenotypes are characteristic of partial loss of function for hlh-1.

f: The host for these injections, PD4251, expresses both mitochondrial GFP and nuclear GFP-LacZ. This allows simultaneous assay for inhibition of gfp (loss of all fluorescence) and lacZ (loss of nuclear fluorescence). The table describes scoring of animals as L1 larvae. ds-gfpG caused a loss of GFP in all but 0–3 of the 85 body muscles in these larvae. As these animals mature to adults, GFP activity was seen in 0–5 additional bodywall muscles and in the eight vulval muscles.

TABLE 2

Effect of injection point on genetic inhibition in injected animals and their progeny

dsRNA	Site of injection	Injected animal phenotype	Progeny Phenotype
None	gonad or body cavity	no twitching	no twitching
None	gonad or body cavity	strong nuclear & mitochondrial GFP	strong nuclear & mitochondrial GFP
unc22B	Gonad	weak twitchers	strong twitchers
unc22B	Body Cavity Head	weak twitchers	strong twitchers
unc22B	Body Cavity Tail	weak twitchers	strong twitchers
gfpG	Gonad	lower nuclear & mitochondrial GFP	rare or absent nuclear & mitochondrial GFP
gfpG	Body Cavity Tail	lower nuclear & mitochondrial GFP	rare or absent nuclear & mitochondrial GFP
lacZL	Gonad	lower nuclear GFP	rare or absent nuclear GFP
lacZL	Body Cavity Tail	lower nuclear GFP	rare or absent nuclear GFP

TABLE 3

C. elegans can respond in a gene-specific manner to environmental dsRNA.

Bacterial Food	Movement	Germline Phenotype	GFP-Transgene Expression
BL21(DE3)	0% twitch	<1% female	<1% faint GFP
BL21(DE3) [fem-1 dsRNA]	0% twitch	43% female	<1% faint GFP
BL21(DE3) [unc22 dsRNA]	85% twitch	<1% female	<1% faint GFP
BL21(DE3) [gfp dsRNA]	0% twitch	<1% female	12% faint GFP

TABLE 4

Effects of bathing *C. elegans* in a solution containing dsRNA.

dsRNA	Biological Effect
unc-22	Twitching (similar to partial loss of unc-22 function)
pos-1	Embryonic arrest (similar to loss of pos-1 function)
sqt-3	Shortened body (Dpy) (similar to partial loss of sqt-3 function)

In Table 2 gonad injections were carried out into the GFP reporter strain PD4251, which expresses both mitochondrial GFP and nuclear GFP-LacZ. This allowed simultaneous assay of inhibition with gfp (fainter overall fluorescence), lacZ (loss of nuclear fluorescence), and unc-22 (twitching). Body cavity injections were carried out into the tail region, to minimize accidental injection of the gonad; equivalent results have been observed with injections into the anterior region of the body cavity. An equivalent set of injections was also performed into a single gonad arm. For all sites of injection, the entire progeny brood showed phenotypes identical to those described in Table 1. This included progeny produced from both injected and uninjected gonad arms. Injected animals were scored three days after recovery and showed somewhat less dramatic phenotypes than their progeny. This could in part be due to the persistence of products already present in the injected adult. After ds-unc22B injection, a fraction of the injected animals twitch weakly under standard growth conditions (10 out of 21 animals). Levamisole treatment led to twitching of 100% (21/21) of these animals. Similar effects were seen with ds-unc22A. Injections of ds-gfpG or ds-lacZL produced a dramatic decrease (but not elimination) of the corresponding GFP reporters. In some cases, isolated cells or parts of animals retained strong GFP activity. These were most frequently seen in the anterior region and around the vulva. Injections of ds-gfpG and ds-lacZL produced no twitching, while injections of ds-unc22A produced no change in GFP fluorescence pattern.

While the present invention has been described in connection with what is presently considered to be practical and preferred embodiments, it is understood that the invention is not to be limited or restricted to the disclosed embodiments but, on the contrary, is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims.

Thus it is to be understood that variations in the described invention will be obvious to those skilled in the art without departing from the novel aspects of the present invention and such variations are intended to come within the scope of the present invention.

We claim:

1. A method to inhibit expression of a target gene in a cell (in vitro) comprising introduction of a ribonucleic acid (RNA) into the cell in an amount sufficient to inhibit expression of the target gene, wherein the RNA is a double-stranded molecule with a first strand consisting essentially of a ribonucleotide sequence which corresponds to a nucleotide sequence of the target gene and a second strand consisting essentially of a ribonucleotide sequence which is complementary to the nucleotide sequence of the target gene, wherein the first and the second ribonucleotide strands are separate complementary strands that hybridize to each other to form said double-stranded molecule, and the double-stranded molecule inhibits expression of the target gene.

2. The method of claim 1 in which the target gene is a cellular gene.

3. The method of claim 1 in which the target gene is an endogenous gene.

4. The method of claim 1 in which the target gene is a transgene.

5. The method of claim 1 in which the target gene is a viral gene.

6. The method of claim 1 in which the cell is from an animal.

7. The method of claim 1 in which the cell is from a plant.

8. The method of claim 6 in which the cell is from an invertebrate animal.

9. The method of claim 8 in which the cell is from a nematode.

10. The method of claim 1 in which the first ribonucleotide sequence comprises at least 25 bases which correspond to the target gene and the second ribonucleotide sequence comprises at least 25 bases which are complementary to the nucleotide sequence of the target gene.

11. The method of claim 1 in which the target gene expression is inhibited by at least 10%.

12. A method to inhibit expression of a target gene in an invertebrate organism comprising:

(a) providing an invertebrate organism containing a target cell, wherein the target cell contains the target gene and the target cell is susceptible to RNA interference, and the target gene is expressed in the target cell;

(b) contacting said invertebrate organism with a ribonucleic acid (RNA), wherein the RNA is a double-stranded molecule with a first strand consisting essentially of a ribonucleotide sequence which corresponds to a nucleotide sequence of the target gene and a second strand consisting essentially of a ribonucleotide sequence which is complementary to the nucleotide sequence of the target gene, wherein the first and the second ribonucleotide sequences are separate complementary strands that hybridize to each other to form the double-stranded molecule; and

(c) introducing the RNA into the target cell, thereby inhibiting expression of the target gene.

13. The method of claim 12 in which the organism is a nematode.

14. The method of claim 13 in which a formulation comprised of the RNA is applied on or adjacent to a plant, and disease associated with nematode infection of the plant is thereby reduced.

15. The method of claim 12 in which said double-stranded ribonucleic acid structure is at least 25 bases in length and each of the ribonucleic acid strands is able to specifically hybridize to a deoxyribonucleic acid strand of the target gene over the at least 25 bases.

16. The method of claim 12 in which the expression of the target gene is inhibited by at least 10%.

17. The method of claim 12 in which the RNA is introduced within a body cavity of the organism and outside the target cell.

18. The method of claim 12 in which the RNA is introduced by extracellular injection into the organism.

19. The method of claim 12 in which the organism is contacted with the RNA by feeding the organism food containing the RNA.

20. The method of claim 19 in which the food comprises a genetically-engineered host transcribing the RNA.

21. The method of claim 12 in which at least one strand of the RNA is produced by transcription of an expression construct.

22. The method of claim 21 in which the organism is a nematode and the expression construct is contained in a plant, and disease associated with nematode infection of the plant is thereby reduced.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,506,559 B1
DATED : January 14, 2003
INVENTOR(S) : Andrew Fire et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Title page,
Item [73] Assignee, should read:
-- [73] Assignee: **The Carnegie Institution of Washington**, Washington, DC (US); **The University of Massachusetts**, Boston, Massachusetts (US) --

Signed and Sealed this

Sixteenth Day of September, 2003

JAMES E. ROGAN
Director of the United States Patent and Trademark Office

Differences Between U.S. Patent No. 6,506,559 B1, issued January 14, 2003 ("Fire et al. Patent") and U.S. Provisional Application No. 60/068,562, filed December 23, 1997 ("Fire et al. Provisional")

- ( ) indicates text which appears only in Fire et al. Patent
- [ ] indicates text which appears only in Fire et al. Provisional
- In Provisional is ". . ." indicates text found in both Fire et al. Patent and Fire et al. Provisional, but with changes in Fire et al. Patent.
- { } gives more specific location of the text in the Provisional, where the text in the Fire et al. Patent does not follow the same order as the Fire et al. Provisional.

1. In Provisional is ":"
2. In Provisional is ","
3. Provisional only

   [, presented in the literature over the last few month,]
4. In Provisional is ". However"
5. Patent only

   (been used to)
6. In Provisional is "inhibited"
7. Patent only

   (The extreme . . . formation)
8. Provisional only

   [These distinctions]
9. Provisional only

   [and non-replicating]
10. Provisional only

    [The process may be practice *ex vivo* or *in vivo*.]

11. Provisional only

[(i.e., a cellular gene)]

12. Provisional only

[(i.e., a cellular gene present in the genome)]

13. Provisional only

[(i.e., a gene construct inserted at an ectopic site in the genome of the cell)]

14. Provisional only

[viral]

15. Patent only

(of a pathogen)

16. Provisional only

[(e.g., at least 10%, 20%, 50%, 75%, 90%, or 95% of targeted cells)]

17. Provisional only

[target gene transcription,]

18. Provisional only

[,]

19. In Provisional is "multiple"

20. Provisional only

[Fully duplex RNA lacks an unpaired region with single-stranded structure.]

21. Provisional only

[(e.g., at least 5, 10, 100, or 1000 copies per cell)]

22. In Provisional is "are"

23. Provisional only

[(see Gribskov and Devereux, *Sequence Analysis Primer*, Stockton Press, 1991, and references cited therein)]

24. Provisional only

    [Greater than 90% sequence identity, or even 100% sequence identity, between the inhibitory RNA and target gene is preferred for the identical nucleotide sequences.]

25. Provisional only

    [(e.g., 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA, 50°C or 70°C for 12-16 hr). The length of the identical nucleotide sequences may be at least 25, 50, 100, 200 or 400 nucleotides]

26. Patent only

    (protozoan . . . or)

27. Provisional only

    [or yeast]

28. In Provisional is "vector"

29. Provisional only

    [(e.g., promoter, enhancer, silencer)]

30. In Provisional is "is"

31. In Provisional is "or"

32. Patent only

    (, introduced . . . affected)

33. In Provisional is "are preferred such as"

34. Patent only

    (directly)

35. In Provisional is "of a solution containing the RNA."

36. Patent only

    (present)

37. In Provisional is "that is"

38. In Provisional is "The present"

39. In Provisional is "the present"

40. In Provisional is "The noted disadvantages"

41. Patent only

(the)

42. Provisional only

[These genes were chosen based on: (1) a defined molecular structure, (2) classical genetic data showing the nature of the null phenotype. Each segment tested for RNAi is designated with the name of the gene followed by a single letter (e.g., *unc22*C). Segments derived from genomic DNA are shown above the gene, segments derived from cDNA are shown below the gene. The consequences of injecting double-stranded RNA segments for each of these genes is described in Table 1. dsRNA sequences from the coding region of each gene produced a phenotype resembling the null phenotype for that gene.]

43. In Provisional is "H"

44. In Provisional is ":"

45. Provisional only

[, both expressed in body muscle. The fluorescent nature of these reporter proteins allowed us to examine individual cells under the fluorescence microscope to determine the extent and generality of the observed inhibition of gene expression]

46. Patent only

(visualized . . . microscope)

47. Provisional only

[These GFP patterns appear identical to the parent strain, with prominent fluorescence in nuclei (the

nuclear localized GFP-LacZ) and mitochondria (the mitochondrially targeted GFP).]

48. Provisional only

[Observable GFP fluorescence is completely absent in over 95% of cells. Only a single active cell is seen in the larva in panel D, while the adult animal in panel E shows staining in none of the striated body wall muscles. Inhibition is not effective in all tissues: the entire vulval musculature expresses active GFP in the adult animal shown in panel E. Panel F shows two rare GFP positive cells in an adult. Both cells express both nuclear-targeted GFP-LacZ and mitochondrial GFP.]

49. In Provisional is ": animals"

50. Provisional only

[In the animals derived from this injection, mitochondrial-targeted GFP appears unaffected while the nuclear-targeted GFP-LacZ is absent from almost all cells (e.g. larva in panel G).]

51. Patent only

((dark stain))

52. Provisional only

[The 1262 nt *mex-3* cDNA clone[20] was divided into two segments, *mex-3*A and *mex-3*B with a short (325 nt) overlap. Similar results were obtained in experiments with no overlap between inhibiting and probe segments. *mex-3*B antisense or dsRNA was injected into the gonads of adult animals, which were fed for 24 hours before fixation and in situ hybridization (see reference 5). The *mex-3*B dsRNA produced 100% embryonic arrest, while

>90% of embryos from the antisense injections hatched. Antisense probes corresponding to *mex-3*A were used to assay distribution of the endogenous *mex-3* mRNA (dark stain). Four-cell stage embryos are shown; similar results were observed from the 1 to 8 cell stage and in the germline of injected adults.]

53. In Provisional is "The scale is such that"

54. Provisional only

[Purified antisense and sense RNA from *unc22*A were injected individually or as an annealed mixture. "Control" was an unrelated dsRNA (*gfpG*). Injected animals were transferred to fresh culture plates 6, 15, 27, 41 and 56 hours after injection. Progeny grown to adulthood were scored for movement in their growth environment, then examined in 0.5 mM levamisole.]

55. Provisional only

[in the graph]

56. Patent only

(Progeny . . . 56 hours The)

57. Provisional only

[:]

58. In Provisional is "showing"

59. Patent only

(FIGs. 5A-C . . . gfp.)

60. In Provisional is "DESCRIPTION OF PREFERRED EMBODIMENTS"

61. Patent only

(producing)

62. Patent only

(by introducing)

63. Provisional only

[with]

64. Provisional only

[¶], *i.e.* a paragraph break

65. Patent only, *but see* Provisional {page 5, line 28 to page 6, line 12}

65.1. Provisional only

[or into the extracellular environment] {page 6, line 1}

65.2. Provisional only

[The process may be practiced ex vivo or in vivo.] {page 6, line 3}

65.3. In Provisional is "viral gene which is present in the cell after infection thereof." {page 6, line 8-9}

65.4. In Provisional is "the procedure" {page 6, line 10}

66. Provisional only

[Sequence]

67. Patent only

(nuclease protection,)

68. Patent only

(gene . . . microarray,)

69. Patent only

((ELISA))

70. Patent only

((RIA), other immunoassays)

71. Patent only

((FACS))

72. Patent only

(acetohydroxyacid synthase (AHAS),)

73. Patent only

((AP))

74. Patent only

((GUS))

75. Patent only

((CAT))

76. Patent only

(horseradish peroxidase (HRP))

77. Patent only

((Luc), . . . (OCS),)

78. In Provisional is "or"

79. Patent only

(Multiple . . . tetracyclin.)

80. Patent only

(as compared . . . invention)

81. Patent only, *but see* Provisional {page 6, line 12 to page 7, line 9}

81.1. Provisional only

[target gene transcription] {page 6, lines 15-16)

81.2. Patent only

(As an example . . . region.)

81.3. In Provisional is "; it" {page 6, line 17}

81.4. Patent only

(For example . . . synthesis.)

81.5. In Provisional is "multiple" {page 6, line 20}

81.6. Provisional only
[Fully duplex RNA lacks an unpaired region with single stranded structure.] {page 6, lines 20 - 21}

81.7. Patent only
(500)

81.8. Patent only
(; lower . . . applications)

81.9. Patent only
(sequence comparison and)

81.10. Patent only
(by, for example, . . . Group).)

81.11. Patent only
(the portion of)

81.12. Provisional only
[for the identical nucleotide sequences] {page 7, line 4}

81.13. Patent only
(hybridization)

81.14. Patent only
(; followed by washing)

81.15. Patent only
(300)

82. Patent only
(As disclose . . . divergence.)

83. Patent only
(protozoan . . . or)

84. Provisional only
[or yeast]

85. Patent only

(Preferred . . . morphologies.)

86. Patent only

(; field . . . bean)

87. Patent only

(cotton . . . sorghum)

88. Patent only

(sunflower,)

89. Patent only

(; vegetable . . . carrot,)

90. Patent only

(celery . . . pepper,)

91. Patent only

(pumpkin . . . juniper;)

92. Patent only

(palm, poplar,)

93. Patent only

(redwood . . . and)

94. In Provisional is "cow"

95. Patent only

(sheep)

96. Patent only

(Representative . . . Homoptera.)

97. In Provisional is "That"

98. Patent only

(having the target gene)

99. Patent only, *but see* Provisional {page 7, line 11 to page 7 line 15}

99.1. In Provisional is "vector" {page 7, line 14}

99.2. Patent only

(, splice . . . polyadenylation)

99.3. In Provisional is "is" {page 7, line 15}

100. Patent only

(Inhibition . . . apparatus.)

101. Patent only

(or enzymatically)

102. Patent only

(a cellular)

103. Provisional only

[of the cell]

104. In Provisional is "construction"

105. In Provisional is "vector"

106. Patent only

([32, 33, 34])

107. Provisional only

[Goeddel, *Gene Expression Technology*, Academic Press, 1990; Kreigler, *Gene Transfer and Expression*, Stockton Press, 1990; Murray, *Gene Transfer and Expression Protocols*, Humana Press, 1991;]

108. Patent only

(also)

109. Patent only

(U.S. Pat. . . . 5,804,693;)

110. In Provisional is "Preferably"

111. In Provisional is "is"

112. Patent only

(and/or . . . strands)

113. Provisional only

[The]

114. In Provisional is "introduced directly"

115. Patent only

(introduced)

116. Patent only

(into)

117. Provisional only

[Examples of extracellular spaces into which the RNA may be introduced include]

118. In Provisional is "or"

119. Patent only

(introduced orally . . . may also be used.)

120. Patent only

(the roots,)

121. Patent only

(the)

122. Patent only

(A transgenic . . . organism.)

123. In Provisional is "are preferred such as for example,"

124. In Provisional is "with"

125. In Provisional is "vector"

126. In Provisional is "vector"

127. In Provisional is "vector"

128. Patent only

(Other methods . . . target gene.)

129. Patent only

(a cell . . . introduced into)

130. Patent only

(carcinogenic/tumorigenic)

131. Patent only

(or maintenance)

132. Patent only

(Treatment . . . envisioned.)

133. Patent only

(or transformed . . . in *C. elegans*)

134. In Provisional is "is"

135. Provisional only

[¶], *i.e.* a paragraph break

136. In Provisional is "can be used"

137. In Provisional is "are"

138. In Provisional is "For instance,"

139. Patent only

(RNA-mediated mechanism was)

140. Patent only

(RNAi)

141. In Provisional is "has"

142. In Provisional is "In particular"

143. In Provisional is "co-suppression"

144. Patent only

(crude)

145. In Provisional is "We"

146. Provisional only

[(see Figure 4)]

147. In Provisional is "interference"

148. Patent only

(While . . . activity)

149. In Provisional is "interfering"

150. In Provisional is "We"

151. Provisional only

[the]

152. Patent only

(of these crude preparations)

153. Provisional only

[below]

154. In Provisional is "and that this subpopulation was"

155. Provisional only

[We disclose that the non-purified RNA populations that were effective in inhibition assays herein include some molecules with double-stranded character.]

156. In Provisional is "we"

157. Patent only

(Analysis . . . Genes)

158. Patent only, *but see* Provisional {page 17, line 3 to page 20, line 23}

158.1. In Provisional is "interference" {page 17, line 11}

158.2. In Provisional is "Table 1" {page 17, line 12}

158.3. In Provisional is "-" {page 17, line 12}

158.4. In Provisional is "-" {page 17, line 15}

158.5. In Provisional is "-" {page 17, line 19}

158.6. In Provisional is "our" {page 18, line 3}

158.7. In Provisional is "-" {page 18, line 3}

158.8. In Provisional is "We found this not" {page 18, line 4}

158.9. Provisional only

[We] {page 18, line 6}

158.10. Patent only

(was)

158.11. In Provisional is "interference" {page 18, line 7}

158.12. In Provisional is "interference" {page 18, line 10}

158.13. Provisional only

[we have only observed] {page 18, line 10}

158.14. In Provisional is "interference" {page 18, line 10}

158.15. Patent only

(was observed only)

158.16. Provisional only

[We assessed] {page 18, line 14}

158.17. In Provisional is "interfere with" {page 18, line 26}

158.18. Patent only

(the)

158.19. Provisional only

[we used] {page 19, line 5}

158.20. Patent only

(was used)

158.21. In Provisional is "we" {page 19, line 11}

158.22. Provisional only

[we saw] {page 19, line 17}

158.23. Patent only

(was noted)

158.24. Provisional only

[we found that] {page 20, line 3}

158.25. In Provisional is "produces" {page 20, line 3}

158.26. Provisional only

[we targeted] {page 20, line 4}

158.27. Patent only
(was targeted)

158.28. Provisional only
[5] {page 20, line 6}

158.29. In Provisional is ". In contrast," {page 20, line 7}

158.30. In Provisional is "D" {page 20, line 9}

158.31. Patent only
(Table 3 . . . 1998.)

158.32. In Provisional is "we" {page 20, line 22}

158.33. Provisional only
[one case] {page 20, line 22}

158.34. Provisional only
[This would not be a consideration for a target gene present in a single copy in the genome.] {page 20, lines 23-24}

159. Patent only
(of)

160. In Provisional is "interference"

161. In Provisional is "interference"

162. Patent only
(Following . . . structure,)

163. In Provisional is "/"

164. In Provisional is "RNA"

165. Patent only
(Methods for)

166. Patent only
(Additional Description of the Results)

167. Patent only, *but see* Provisional {page 8, line 10 to page 10, line 12}

167.1. Patent only

(sequence . . . follows:)

167.2. In Provisional is "RNAi" {page 8, line 15}

167.3. In Provisional is "Figure 2A-H show analysis" {page 8, line 21}

167.4. Patent only

(were analyzed)

167.5. Provisional only

[effects] {page 8, line 21}

167.6. Patent only

((FIG. 2, panels A-H))

167.7. Patent only

(ds-unc22A . . . affected.)

167.8. Provisional only

[The micrographs show progeny of injected animals. Panel A (young larva), B (adult), and C (adult body wall; high magnification) result from injection of a control RNA (*ds-unc22*A).] {page 8, lines 26-28}

167.9. In Provisional is "These" {page 8, line 28}

167.10. Patent only

(of the progeny)

167.11. In Provisional is "appear" {page 8, line 28}

167.12. Patent only

(: young larva . . .(FIG. 2C).)

167.13. Patent only

(In contrast, the)

167.14. Provisional only

[Panels D-F show] {page 9, line 1}

167.15. Patent only

(RNA . . . (FIGS. 2D-F).)

167.16. Patent only

(Few . . . muscle cells).)

167.17. Provisional only

[Only a single active cell is seen in the larva in panel D, while the adult animal in panel E shows staining in none of the striated body wall muscles.] {page 9, lines 3-4}

167.18. In Provisional is "is" {page 9, line 4}

167.19. In Provisional is "expresses" {page 9, line 5}

167.20. In Provisional is "shown in panel E." {page 9, line 6}

167.21. Provisional only

[Panel F shows two] {page 9, line 6}

167.22. Patent only

(were also seen)

167.23. In Provisional is "in an" {page 9, line 6}

167.24. Patent only

(animals . . . FIG 2F).)

167.25. In Provisional is "Panels G-I demonstrate specificity:" {page 9, line 7}

167.26. In Provisional is "are" {page 9, line 8}

167.27. In Provisional is "is" {page 9, line 11}

167.28. Provisional only

[e.g.,] {page 9, line 11}

167.29. In Provisional is "Panel H shows a typical adult, with" {page 9, line 11}

167.30. Provisional only
[lacking] {page 9, line 12}

167.31. Patent only
(activity)

167.32. Patent only
((FIG. 2H))

167.33. Patent only
(in FIG. 2)

167.34. Provisional only
[Figures 3 A-D show] {page 9, line 14}

167.35. Patent only
(The)

167.36. Patent only
(was show . . . panels A-D).)

167.37. Provisional only
[Micrographs show *in situ* hybridization to embryos.] {page 9, line 15}

167.38. In Provisional is "fed" {page 9, line 19}

167.39. In Provisional is "are shown;" {page 9, line 23}

167.40. In Provisional is "Panel A: Negative control showing lack of staining in the absence of hybridization probe. Panel B: Embryo from uninjected parent (normal pattern of endogenous mex-3 RNA^{20}). Panel C: Embryo from a parent injected with purified mex-3B antisense RNA. These embryos and the parent animals retain the mex-3 mRNA, although levels may have been somewhat less than wild type. Panel D: Embryo from a parent injected with

dsRNA corresponding to mex-3B; no mex-3 RNA was detected." {page 9 line 14 - page 10, line 1}

167.41. Patent only
(of FIG. 3)

167.42. In Provisional is "Figure 4 shows" {page 10, line 3}

167.43. In Provisional is "of" {page 10, line 3}

167.44. Patent only
(was measured)

167.45. Patent only
(RNA)

167.46. Patent only
((FIG. 4))

167.47. In Provisional is "6, 15, 27, 41, and 56 hours" {page 10, line 6}

167.48. Patent only
(The)

167.49. Provisional only
[:] {page 10, line 10}

167.50. In Provisional is "showing" {page 10, line 10}

167.51. Patent only
(the)

168. Patent only
(FIG. 5A-C . . . effective.)

169. Patent only, *but see* Provisional {page 20, line 25 to page 20, line 25}

169.1. Patent only
(references (e.g.,)

169.2. Patent only

(application,)

169.3. Patent only

(indicative . . . disclosures are)

170. In provisional is "Reference"

171. In Provisional is "Karn"

172. Patent only

(28 . . . 1991.)

173. In Provisional is "layed" {page 24, line 10}

174. Patent only

(In Table 2,)

175. Provisional only

[present] {page 26, line 22}

176. Patent only

(New Table 3 added)

177. Patent only

(in vitro)

178. In Provisional is "has" {page 27, line 5}

179. Provisional only

[structure with an identical nucleotide sequence as compared to a portion of the target gene.] {page 27, lines 5-6}

180. Patent only

(molecule . . . target gene.)

181. Provisional only

[identical nucleotide sequence is at least 50 nucleotides in length.] {page 27, lines 24-25}

182. Patent only

(first ribonucleotide . . . target gene.)

183. In Provisional is

"12. The method of claim 1 in which the cell is present in an organism and inhibition of target gene expression demonstrates a loss-of function phenotype.

13. The method of any one of claims 1-12 in which the RNA has one self-complementary strand.

14. The method of any one of claims 1-12 in which the RNA has two separate complementary strands.

15. The method of claim 14 further comprising synthesis of the two complementary strands and initiation of RNA duplex formation outside the cell.

16. The method of claim 14 further comprising synthesis of the two complementary strands and initiation of RNA duplex formation inside the cell.

17. The method of any one of claims 1-12 in which the RNA has no single stranded structure.

18. The method of any one of claims 1-12 in which the RNA is introduced within the body cavity of an animal and outside the cell.

19. The method of any one of claims 1-12 in which the RNA is introduced by extracellular injection into a body cavity of an organism.

20. The method of any one of claims 1-12 in which an expression vector in a cell produces the RNA." {page 28, lines 1-26}

184. Patent only

(Labels "FIG. 2", "CONTROL RNA (ds-*unc22a*)", "ds-*gfpG* RNA", "ds-*laxZL* RNA", "L1" and "ADULT" are added to the graphs in Figure 2)

185. Patent only

(Label "FIG. 3" is added to the caption for each graph in Figure 3)

186. Patent only

(Labels "FIG. 4A" and "FIG. 4B" are added)

187. Provisional only

[Section labeled "Progeny Cohort Group"]

188. Patent only

(New Figure 5 added)

EXHIBIT C

30 min at 37°C, either with or without preheating at 90°C for 5 min, followed by electrophoresis in a 15% denaturing gel.

For detection of *rev*-EGFP mRNA, we used a 25-mer deoxyribonucleotide probe that was complementary to the EGFP mRNA of the *rev*-EGFP fusion protein. A 29-mer deoxyribooligonucleotide probe was used for detection of the GAPDH transcript.

HIV-1 antiviral assay. For determination of anti-HIV-1 activity of the siRNAs, transient assays were done by cotransfection of siDNAs and infectious HIV-1 proviral DNA, pNL4-3 into 293 cells as described[15]. Before transfection, the cells were grown for 24 h in six-well plates in 2 ml EMEM supplemented with 10% (vol/vol) FBS and 2 mM L-glutamine, and transfected using Lipofectamine Plus reagent (Life Technologies, GibcoBRL) as described by the manufacturer. The DNA mixtures consisting of 0.5 μg siDNAs or controls, and 0.5 μg pNL4-3 were formulated into cationic lipids and applied to the cells. After one, two, three, and four days, supernatants were collected and analyzed for HIV-1 p24 antigen (Beckman Coulter, Hialeah, FL). The p24 values were calculated with the aid of the Dynatech MR5000 ELISA plate reader (Dynatech Labs Inc., Chantilly, VA). Cell viability was also assessed using a Trypan Blue dye exclusion count at four days after transfection.

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Competing interests statement

The authors declare competing financial interests: see the Nature Biotechnology *website (http://biotech.nature.com) for details.*

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Effective expression of small interfering RNA in human cells

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In many eukaryotes, expression of nuclear-encoded mRNA can be strongly inhibited by the presence of a double-stranded RNA (dsRNA) corresponding to exon sequences in the mRNA (refs 1,2). The use of this "RNA interference" (RNAi) in mammalian studies had lagged well behind its utility in lower animals because uninterrupted RNA duplexes longer than 30 base pairs trigger generalized cellular responses through activation of dsRNA-dependent protein kinases[3]. Recently it was demonstrated[4] that RNAi can be made to work in cultured human cells by introducing shorter, synthetic duplex RNAs (~20 base pairs) through liposome transfection. We have explored several strategies for expressing similar short interfering RNA (siRNA) duplexes within cells from recombinant DNA constructs, because this might allow long-term target-gene suppression in cells, and potentially in whole organisms. Effective suppression of target gene product levels is achieved by using a human U6 small nuclear RNA (snRNA) promoter to drive nuclear expression of a single RNA transcript. The siRNA-like parts of the transcript consists of a 19–base pair siRNA stem with the two strands joined by a tightly structured loop and a U_{1-4} 3′ overhang at the end of the antisense strand. The simplicity of the U6 expression cassette and its widespread transcription in human cell types suggest that this mode of siRNA delivery could be useful for suppressing expression of a wide range of genes.

The U6 snRNA promoter cassettes and si-like RNA inserts are shown in Figure 1. We previously showed that RNA expressed by RNA polymerase III from the U6+1 or U6+27 cassettes was expressed primarily as full-length transcripts and was located in the nucleus[5,6]. U6+27 transcripts, containing the first 27 nucleotides of human U6 RNA, were capped with γ-methyl phosphates and accumulated to higher levels than U6+1 transcripts. Cassettes are designed so that short RNA coding sequences are inserted between unique *Sal*I and *Xba*I sites. After the *Xba*I site, the cassette encodes a strong stem to protect the transcripts against 3′-5′ exonuclease attack, then a poly(U) transcription termination sequence. However, the insertion sequences discussed later also contain their own UUUU terminator at the 3′ end of the inserted sequences, terminating most transcription before the cassette-encoded stem/terminator region.

To test whether expressed si-like RNA is effective, we targeted a site in human lamin A/C mRNA that has been demonstrated to be vulnerable to synthetic siRNA[4]. The inserted sequences encoded several variants of siRNA duplexes and controls, shown in Figure 1B. Previous work on synthetic anti-lamin A/C siRNA used two independent strands with 3′ unpaired tails[4]. Although it would be theoretically possible to synthesize two strands independently *in vivo*, the need to anneal the two strands could make the production

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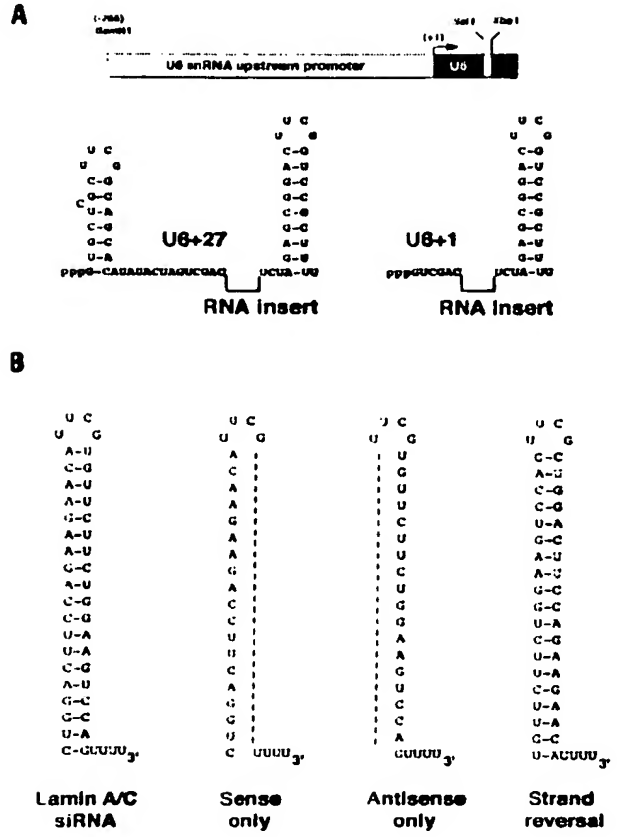

Figure 1. Expression cassettes and small RNA inserts. (A) The two U6 snRNA promoter[12–14] expression cassettes used to express siRNAs and controls are shown with the expected transcripts by RNA polymerase III, assuming no UUUU terminators in the RNA insert. Cassettes had either no remaining U6 snRNA sequences (U6+1) or the first 27 nucleotides of U6 snRNA (U6+27) to direct methylation of the 5′-γ-phosphate and stabilize the transcript[5]. With the inserts shown, most transcription terminates with the insert UUUU, but readthrough to the cassette stem terminator also occurs. (B) Four tested anti-lamin RNA inserts are shown. Each would begin immediately after the *Sal*I sequence from the cassette, and most termination occurs after the UUUU at the insert 3′ terminus (ref. 5 and data not shown).

of siRNA inefficient, and might complicate routine cloning and expression of the siRNA constructs. However, as seen elsewhere in this issue (Lee *et al.*, p. 500), the synthesis of the siRNA as independent strands from U6 promoter can also be effective.

To make the siRNA duplex as one short transcript, an RNA insert was used that contains the 19-nucleotide sense strand of the target, followed by a UUCG tetraloop sequence[7], the antisense strand, and a UUUU transcription terminator, in that order. This terminates a high percentage of the transcripts exactly at the end of the siRNA stem[5]. The 3′-UUUU overhang after the siRNA is attacked by 3′ exonucleases, leaving 1 to 4 U 3′-end overhangs[5]. Results with synthetic siRNA[4] suggest that such 3′ overhangs can increase efficacy. RNA blot analysis has shown that high levels (~10^4–10^5 RNA molecules/cell) of nearly full-length RNAs can be expressed from these cassettes[5]. The "hairpin siRNAs" give comparable expression levels, although there is a complex pattern of breakdown products as well as the full-length product (not shown).

Figure 2 shows elimination of lamin A/C protein when HeLa cells were transiently transfected with either synthetic siRNA or siRNA-expressing clones. Cells shown in Figure 2A underwent Oligofectamine-mediated transfection with either no RNA or a synthetic 19–base pair siRNA duplex with 3′-TT overhangs[4]. Nuclei in Figure 2A were visualized with 4,6-diamidino-2-phenylindole (DAPI) staining (blue). As expected from previous work, the lamin A/C signal (red) substantially disappears from most cells, presumably those that are transfected. For testing recombinant DNAs, cells were cotransfected with a plasmid (pCMVβ) expressing β-galactosidase (β-Gal) to mark transfected cells. Production of β-Gal also precludes the possibility that siRNA constructs nonspecifically obstruct protein synthesis. Figure 2B shows individual frames with staining for lamin A/C (red), β-Gal (green), or the overlay of the two signals. Without the siRNA inserts, cells transfected with any of the expression cassette plasmids do not have detectably reduced lamin A/C signal (shown only for U6+1 in Fig. 2B). When either U6+1 or U6+27 cassettes were used with anti-lamin hairpin siRNA inserts, dramatic reductions of lamin A/C signal were observed relative to the untransfected cells in the same fields. Transfected cells receiving the U6+27-siRNA expression cassettes gave the most consistent and greatest lamin A/C reductions (>90%, Table 1), similar to synthetic siRNA (~95%). This might reflect a threshold effect caused by lower levels of the U6+1-expressed siRNA[5].

Figure 2C shows lamin A/C–β-Gal overlay panels for control RNAs expressed from the U6+27 cassette. Expression of only the sense or only the antisense strands of the siRNA in U6+27 did not affect lamin levels, reinforcing the notion that the observed reduction in Figure 2B requires the duplex, a hallmark of siRNA action.

We next tested a U6+27 hairpin siRNA construct with the order of the strands reversed to determine the specific need for an accessible 3′ overhang on the antisense strand of the duplex. Some models for siRNA function predict that siRNA degradation of the target message is amplified by annealing of the antisense strand to the mRNA and extension to a longer duplex with an RNA-dependent RNA polymerase. This condition would indicate the need to have an accessible antisense 3′ terminus so that it can be extended. Surprisingly, there was a significant reduction of the lamin signal with the reversed-strand construct, although it was not as consistent or effective as the original orientation. It is not clear why the reversed-strand construct causes partial reduction of the lamin signal. It is possible that small amounts of breakdown products with 3′-UU overhang are created on the antisense strand of the reversed construct by 3′ exonuclease digestion or a discrete endonuclease cleavage between the strands. Alternatively, these hairpin siRNAs, when expressed within the cells, might not need to act exclusively through primer extension amplification. Although the active form of the nuclear-expressed RNAs will require long-term investigation, we recommend that siRNA transcripts have the sense strand first, followed by a tetraloop and antisense strand ending with a 3′ overhang created by the poly(U) terminator.

Previous studies of siRNA-mediated target cleavage by extracts *in vitro* suggested that the 5′ termini of one or both strands might need to be phosphorylated, and that this might be needed for efficient assembly into obligatory ribonucleoprotein complexes[8,9]. Results pre-

Table 1. Effect of siRNA and expression cassettes on the levels of the lamin A/C protein in transfected cell nuclei

Construct	Percentage lamin A/C in transfected vs. nontransfected cells
pAVU6+27 No insert	130 ± 5
Synthetic anti-lamin siRNA	5 ± 2
pAVU6+27 Anti-lamin siRNA hairpin	9 ± 5
pAVU6+27 Sense strand only	130 ± 40
pAVU6+27 Antisense strand only	130 ± 30
pAVU6+27 Reverse-strands hairpin	25 ± 14

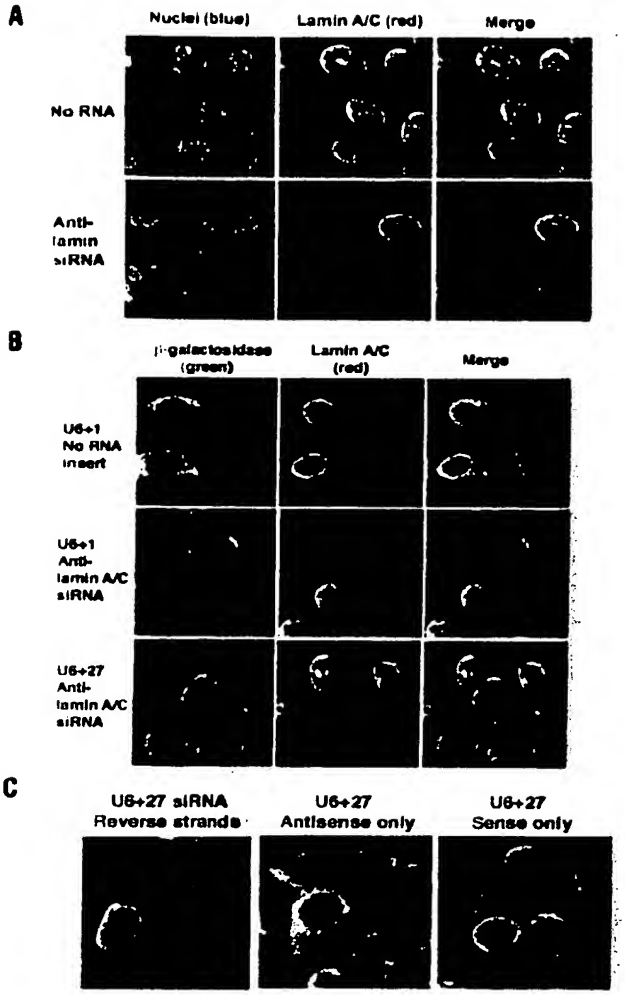

Figure 2. Effects of siRNA constructs on lamin A/C levels. HeLa cells were transfected with either synthetic siRNA or recombinant DNA cassettes expressing different small RNAs from different RNA polymerase III promoters. Cells were stained with DAPI (blue) or with antibodies to lamin A/C (red) or β-Gal (green). (A) Synthetic siRNA or no RNA transfections, showing that lamin A/C staining of the nuclear periphery is largely abolished in most cells, with only low levels of residual red staining in nuclear interiors. (B) Transfection with U6 promoter cassettes either without an siRNA insert (U6+1, no insert) or containing the anti-lamin siRNA shown in Figure 1B (U6+1 siRNA and U6+27 siRNA). Transfected cell cytoplasms are green, whereas nuclei from untransfected cells show no green cytoplasm. Empty expression cassettes have no apparent effect on lamin A/C levels (only empty U6+1 is shown), while transfected cells (green) using siRNA-expressing constructs have little remaining lamin A/C (red). (C) Overlay panels of β-Gal and lamin A/C signal after transfection with different control insertions shown in Figure 1B. Quantitative assessment of remaining lamin A/C signals in transfected cells compared to untransfected cells on the same slide is given in Table 1.

sented here suggest that the hairpin siRNAs might not need to have the 5′ end of either strand unblocked. These hairpin siRNA observations would be consistent with a mechanism in which the U6 transcript containing the hairpin duplex RNA is able to assemble into any necessary protein complexes. The moderate preference for the antisense strand at the end agrees with the prediction that the antisense strand is used as a primer for RNA-dependent RNA polymerase on the message target[10], but this appears not to be essential.

Another unexpected finding was that expression from the U6 snRNA promoter cassettes, which give primarily nucleoplasmic expression[5], would succeed in inhibiting target expression when a majority of the existing mRNA is cytoplasmic. To be certain that the long hairpin did not cause altered localization, we carried out *in situ* hybridization with fluorescent probes to the hairpin that showed nuclear localization very similar to that seen previously (Fig. 3). Admittedly, a small percentage of the U6-driven transcripts might exit to the cytoplasm and be active there. These results suggest that the U6-driven transcripts are suppressing pre-mRNAs before nuclear exit.

The U6 expression cassettes used in these studies are <400 base pairs long and should be relatively easy to incorporate into a variety of vectors. The siRNA inserts can be synthesized as complementary oligodeoxynucleotide pairs to rapidly create cassettes directed at multiple sites. It should even be possible to use several cassettes per vector, targeted at either multiple mRNAs or multiple sites on the same message. It is likely that the hairpin siRNA strategy will be applicable to many mRNA targets. Preliminary experiments targeting both an endogenous human splicing factor and HIV-1 reverse transcriptase coding region (A. Ehsani, S. Li, A. Kleihauer, and J.J. Rossi, personal communication) have shown the hairpin siRNA strategy to be effective. However, as with the synthetic siRNAs, it is sometimes necessary to test several target sites along an mRNA to find one that gives the strongest inhibition. While much remains to be learned about the mechanism by which these transcripts work, the results with the simple U6 cassettes suggest that they might be useful for diverse experimental applications.

Experimental protocol

Materials. Lipofectin, Plus reagent, and Oligofectamine were purchased from Invitrogen (Carlsbad, CA), as were synthetic DNA oligonucleotides for cloning and probes. Cy3-2′-*O*-methyl RNA oligonucleotide hybridization probes were from Trilink (San Diego, CA). Synthetic siRNA oligonucleotides were from Dharmacon (Lafayette, CO). Anti-lamin A/C monoclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) (sc-7292, used at 1 μg/ml); rabbit anti-β-Gal antibodies were from Molecular Probes (Eugene, OR) (A-11132, 1 μg/ml); Oregon green 488–labeled goat anti-rabbit secondary antibodies were from Molecular Probes (O-11038, 5 μg/ml); and cyanin-3 (Cy3)-labeled goat anti-mouse secondary antibodies were from Amersham-Pharmacia Biotech (Piscataway, NJ) (PA 43002,

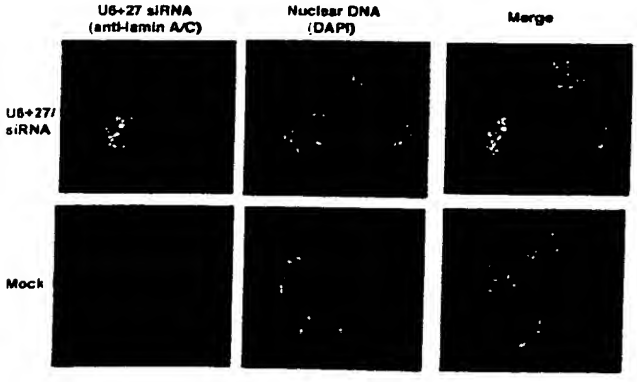

Figure 3. Localization of U6+27 siRNA transcripts. Two days after transfection with the U6+27 anti-lamin A/C cassette plasmid, cells were fixed and stained for nuclear DNA (DAPI, blue) and probed with a Cy3-labeled 2′-O-methyl oligoribonucleotide (red) complementary to the antisense strand of the siRNA. As expected from work with previous U6 expression constructs[5,6], the U6+27 siRNA pattern was primarily in a nuclear speckled pattern. Nuclear and cytoplasmic background staining by the Cy3-oligonucleotide in the absence of U6+27-siRNA ("Mock") was minimal.

1 μg/ml). Cassettes[3] were cloned in pAV vectors, derived from pCWRSVN (ref. 11) by placing the promoter modules between *Bam*HI and *Hin*dIII sites, after modifying the vector. Modifications included destruction of the *Bam*HI site downstream of the Neo cassette, and removal of all sites between the original *Sal*I and *Xho*I sites, inclusive, by cleavage and religation. After inserting the cassettes, a new polylinker was created between the *Hin*dIII and *Sac*II sites. Sequences to be expressed were inserted as synthetic oligodeoxynucleotides precisely between the end of the unique *Sal*I site and the beginning of the unique *Xba*I site. Recombinant constructs were sequenced.

Transfections. Transient transfections were carried out on subconfluent HeLa cells. Synthetic RNA was transfected using Oligofectamine as described[4]. Recombinant DNA constructs were transfected using Lipofectin with Plus reagent according to the manufacturer's instructions. In transient transfections, cells were split after one day. Cells were fixed and examined for lamin protein after three days, and fixed and examined by *in situ* hybridization after two days.

Fluorescence microscopy. Transfected cells were fixed and subjected to previously described protocols for visualizing proteins[4] with antibodies (lamin A/C and β-Gal) or detecting small RNAs (http://singerlab.aecom.yu.edu/protocols) by hybridizing 5′-Cy3-labeled oligos (5′-Cy3-AAACUGGACUUCCAGAAGAACACGAA, 2′-*O*-methyl ribonucleotides) to the fixed preparations. Fluorescence was acquired with a Nikon Eclipse E800 (Tokyo, Japan) with a Hamamatsu Orca II camera (Hamamatsu-City, Japan). For each construct, hundreds of cells were examined to confirm that the selected images were representative. On multiple slides, lamin A/C fluorescence in transfected cells was deconvoluted and quantitated using Isee software (Inovision; Raleigh, NC) and is expressed in Table 1 as a percentage of lamin A/C signal from nontransfected cells on the same slides. Lamin signal was consistently higher in transfected cells than in untransfected cells on the same slide.

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Competing interests statement

The authors declare competing financial interests: see the Nature Biotechnology *website (http://biotech.nature.com) for details.*

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Using the transcriptome to annotate the genome

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A remaining challenge for the human genome project involves the identification and annotation of expressed genes. The public and private sequencing efforts have identified ~15,000 sequences that meet stringent criteria for genes, such as correspondence with known genes from humans or other species, and have made another ~10,000–20,000 gene predictions of lower confidence, supported by various types of *in silico* evidence, including homology studies, domain searches, and *ab initio* gene predictions[1,2]. These computational methods have limitations, both because they are unable to identify a significant fraction of genes and exons and because they are unable to provide definitive evidence about whether a hypothetical gene is actually expressed[3,4]. As the *in silico* approaches identified a smaller number of genes than anticipated[5–9], we wondered whether high-throughput experimental analyses could be used to provide evidence for the expression of hypothetical genes and to reveal previously undiscovered genes. We describe here the development of such a method—called long serial analysis of gene expression (LongSAGE), an adaption of the original SAGE approach[10]—that can be used to rapidly identify novel genes and exons.

The LongSAGE method (Fig. 1) generates 21 bp tags derived from the 3′ ends of transcripts that can rapidly be analyzed and matched to genomic sequence data. The method is similar to the original SAGE approach[10], but uses a different type IIS restriction endonuclease (*Mme*I) and incorporates other modifications to produce longer transcript tags. The resulting 21 bp tag consists of a constant 4 bp sequence representing the restriction site at which the transcript was cleaved, followed by a unique 17 bp sequence derived from an adjacent sequence in each transcript. Theoretical calculations show that >99.8% of 21 bp tags are expected to occur only once in genomes the size of the human genome (Table 1A). Likewise, similar analyses based on actual sequence information from ~16,000 known genes suggest that >75% of 21 bp tags would be expected to occur only once in the human genome, with the remaining tags matching duplicated genes or repeated sequences (as discussed below). In contrast, conventional SAGE tags of 14 bp do not allow unique assignment of tags to genomic sequences, though they do allow such assignment to the much less complex compendium of expressed sequence tags (ESTs) and previously characterized mRNAs[10–12]. To optimize the quantification of transcripts, tags are ligated together to form "ditags," which are then concatenated and cloned. Sequencing tag concatemers in parallel allows the identification of up to ~30 tag sequences in each sequencing reaction. Matching tags to genome

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